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EVALUATION OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY FOR DETECTION OF *Theileria parva* IN CATTLE IN APAC DISTRICT

BY

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DECLARATION

I, **Wandera Julius,** declare that the work presented here is my original work and has never been submitted to any university for the award of any degree.

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DEDICATION

To my beloved wife, Mrs. Ssanyu Winfred Nakalema, my children Jed Gada Nyafwono, Jadyn Wandera Alow, Janis Kisan and Joash Jeremiah Owere, my brother Owere Hannington, my father Owere Wycliffe and my Late mother Hadija Modoi Namuyeyi (RIP).

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LIST OF SYMBOLS, ACRONYMS AND ABBREVIATIONS

%:	percentage
µl:	microlitre
µM:	microMolar
^{o}C:	Degrees Centigrade
AEZ	Agricultural Economic Zones
B3:	Backward outer primer
BIP:	Backward inner primer
bp:	Base pair
CD:	Cluster of differentiation
CFA:	Complement Fixation Assay
CI:	Confidence intervals
cM:	centeMolar
COVAB:	College of Veterinary Medicine, Animal Resources and Biosecurity
DNA:	Deoxyribonucleic Acid
ECF:	East Coast fever
EDTA:	Ethylene diaminetetraacetic acid
ELISA:	Enzyme-Linked Immunosorbent Assay
et al:	and others
F3:	Forward outer primer
fg:	femtogram
FIP:	Forward inner primer
IFAT:	Indirect Fluorescent Antibody Test
IgG:	Immunoglobulin G
IL:	Interleukin
ILRI:	International Livestock Research Institute
ITM:	Infection and Treatment Method
Kb:	Kilo base
KDa:	Kilo Dalton
LAMP:	Loop-mediated isothermal amplification

LB:	Loop backward primer
LF:	Loop forward primer
MHC:	major histocompatibility complex
ml:	millilitres
mM:	millimolar
NC:	Negative Control
ng:	Nanogram
NPV:	Negative predictive value
nPCR:	nested Polymerase Chain Reaction
OIE:	Office International des Epizooties
PBS:	Phosphate Buffered Saline
PC:	Positive Control
PCR:	Polymerase Chain Reaction
PIM:	Polymorphic Immunodominant Molecule
pM:	picoMolar
PPV:	Positive predictive value
RBC:	Red blood cell
RLB:	Reverse Line Blot
RNA:	Ribonucleic acid
rRNA:	ribosomal Ribonucleic acid
rpm:	revolutions per minute
rtPCR:	real time Polymerase Chain Reaction
SNP:	Single-nucleotide polymorphism
SSU:	Small subunit
TAE:	Tris-Acetate Ethylene diaminetetraacetic acid
TBDs:	Tick borne diseases
USD:	United States of America Dollar
UV:	Ultraviolet
VNTR:	Variable number of tandem repeats
WHO:	World Health Organization

DEFINITION OF TERMS

Asymptomatic

This is an individual that has been infected with pathogen causing organism but is not showing any signs or symptoms of the illness. Symptoms of the disease usually appear in later stages of the disease.

Carrier State

This is a condition in which an individual or cattle that is not sick but harbors an infective organism which may cause disease in those to whom it is transmitted.

Isothermal amplification

This is a nucleic acid testing technique that rapidly and efficiently accumulates nucleic acid sequences at constant temperature.

Diagnostic accuracy

This is the ability of a test to correctly distinguish between the presence and absence of a disease or condition. It is a measure of how well a diagnostic test performs in identifying true positives and true negatives.

Screening test

This is a test, or a procedure performed on an individual who does not show any symptoms of a particular disease, with the aim of detecting the disease or condition early.

ABSTRACT

East Coast fever (ECF) is an economically important tick-borne disease that affects cattle in East, Central and Southern Africa. The disease is caused by the parasite called *Theileria parva* whose main vector is the tick known as *Rhipicepahalus appendiculatus*. Control of ECF involves the use of acaricides, physical barriers to limit tick access, immunization using the Muguga cocktail and theilericidal drugs. Early detection of *T. parva* and timely treatment are important for controlling ECF. The Loop mediated isothermal amplification (LAMP) assay, which utilizes a single constant temperature for DNA amplification as opposed to the classical PCR method, was previously developed by Eiken Chemicals Co. Ltd. This study evaluated the LAMP assay as a diagnostic test to detect *T. parva* in ECF endemic areas. The performance (sensitivity, specificity and accuracy) of LAMP was evaluated using p104-nPCR as reference standard. A total of 100 cattle blood samples were systematically selected from 1726 archived blood samples previously collected from Apac district during a broader cross-sectional study on Animal African Trypanosomiasis in northern Uganda. Genomic DNA material was extracted from the cattle blood samples and amplification was achieved with PIM gene primers for LAMP assay and the p104 gene primers for nPCR. The *T. parva* amplicons were visualized by SYBR green 1 dye for LAMP and confirmed by gel electrophoresis for nPCR (n=21) and LAMP (n=24) positive cattle blood samples. Statistical analysis by MedCalc software observed specificity of 96.2%, sensitivity of 100% and diagnostic accuracy of 97% for the LAMP assay in relation to nPCR. The positive predictive value and negative predictive value were 87.5% and 100% respectively, and the level of concordance between the PIM-LAMP assay and the p104-nPCR was “almost perfect” (k-value: 0.91). Thus, PIM-LAMP assay is a simple yet sensitive, specific and accurate diagnostic tool for ECF. With the use of simple DNA crude extraction techniques and fluorescence dye, the LAMP assay could be considered for detections of infections caused by *T. parva* in the laboratory-resource-limited endemic areas.

CHAPTER ONE

INTRODUCTION

1.1 Background

East Coast fever (ECF) is an important cattle disease in tropical and subtropical Africa that leads to significant economic losses to farmers through morbidity and mortality of cattle (Muhanguzi *et al*., 2014). The disease is caused by a protozoan parasite, *Theileria parva,* which is transmitted by the brown ear tick, *Rhipicephalus appendiculatus* (Byaruhanga *et al*., 2015). The parasite infects and transforms lymphocytes, causing severe and often fatal lymphocytosis (McKeever, 2009). *Theileria parva* is hosted in cattle, amongst recovered host animals in a carrier state and naturally by the African Cape buffalo (*Syncerus caffer*) (Bishop *et al*., 2004). The prevalence of *T. parva* in cattle in Uganda is highly variable depending on the region, farming system, and control measures used, an overall prevalence of 30%, with prevalence rates varying significantly across different agro-ecological zones (AEZs) was reported by Kabi *et al* (2014). In Iganga district, eastern Uganda, prevalence of 46.8% was reported (Oliga *et al.,* 2023) and Billiou *et al* (2005) reports prevalence *T. parva* of 26% using RLB and 43% using p104 PCR covering central and eastern Uganda. Similarly, ECF prevalence using conventional PCR method was reported as 19.8% and 5.3% Mbarara and Tororo districts respectively (Muhanguzi *et al.*, 2015), indicating the significance of diagnostic method in ECF prevalence and surveillance programmes.

Effective control of theileriosis involves the use of tick control methods like restriction of cattle movement, tick picking and application of acaricides. Prevention tagged with tick control, immunization and treatment using anti-theilericidal drugs like buparvaquone (Muhanguzi *et al*., 2014) has managed to reduce the effects of ECF in cattle populations. Theileriosis control is strongly measured by the diagnostic methods used (Muhanguzi *et al.*,2015). Field diagnosis of ECF using clinical signs is often followed by microscopy as the standard method for field application. Despite being highly specific and inexpensive, microscopy has been reported to be less sensitive, requires high parasitemia and microscopy expertise (Zweygarth *et al*., 1997; Carelli *et al*., 2007). By contrast, serological methods like Enzyme-linked immunosorbent assay (ELISA), sensitivity and specificity of 90% and Indirect fluorescence antibody test (IFAT), sensitivity of

90% and specificity of 80% are more sensitive than microscopy (Muraguri *et al.,* 1999). The ELISA test and IFAT, methods in detection *T. parva* demonstrated a low agreement with Kappa value of 0.21 (Muraguri *et al*.,1999). Serological diagnostic methods are highly sensitive and specific to *T. parva* than microscopy, though they detect historical infections, are expensive to execute and require well-resourced laboratory. Molecular based methods have been developed to detect *T. parva* and offer superior sensitivity to serological and microscopic methods. Noaman *et al* (2014) reported a sensitivity of 57% and specificity of 99%, for microscopy and a sensitivity and specificity of 100% for PCR. A comparison of Reverse Line Blot (RLB) and β-tubulin-targeted nPCR for the detection of Theileria and Babesia reported an agreeable level of concordance with a Kappa value of 0.79 (Atuhaire *et al.,* 2013). Despite microscopy being field applicable, low cost and simpler procedure to execute, *T. parva* is increasingly being diagnosed by p104 nPCR (Odongo *et al.,* 2010) and β-tubulin targeted nPCR (Atuhaire *et al.,* 2013). Thekisoe *et al* (2010) developed a LAMP method targeting p150 and PIM genes of *T. parva,* using the 18S RNA gene PCR for reference. Despite being highly sensitive and specific, p150 and PIM-LAMP assays require more comparative data for validation. The polymorphic immunodominant molecule (PIM) gene contains regions that are both highly conserved across different geographic isolates of the parasite *T. parva* and specific to that species due to its polymorphism (Thekisoe *et al.,* 2010). The Loop mediated isothermal amplification assays offers quick turnaround time, high diagnostic accuracy, and use of a single temperature for amplification. The long term cost of LAMP assay was found to be comparable to microscopy method in the detection of malaria parasite (Zelman *et al*., 2018). Therefore, with the advantages of LAMP and the shortcomings of microscopy, LAMP assay offers the opportunity to be deployed as a field diagnostic tool within a moderately resourced laboratory. A fully operational LAMP assay will be able to provide confirmatory results of *T. parva* infection in cattle during surveillance programmes, allowing for the rapid application of interventions like treatment, isolation, and immunization.

1.2 Problem statement

A Loop-mediated isothermal amplification assay targeting a *T. parva* PIM gene (PIM-LAMP) has the potential to offer a simple, rapid, and visual result interpretation, making it suitable for field result applications. However, the diagnostic performance (sensitivity, specificity and accuracy) of the PIM-LAMP assay in detecting *T. parva* particularly submicroscopic or carrier-state infection

common in endemic areas, needs to be rigorously evaluated and validated against the established nPCR standard using field samples. There is a lack of comparative data to confidently implement the PIM-LAMP assay as a reliable field applicable tool despite its obvious advantage of accurate and timely results that would otherwise mitigate the challenge of misdiagnosis, late treatment and cattle mortality.

1.3 General objective

To evaluate the diagnostic performance of PIM-LAMP assay for detection of *T. parva* infection in cattle, using p104-nPCR as 'gold standard'.

1.3.1 Specific objectives

(i) To determine the diagnostic accuracy, specificity and sensitivity of PIM-LAMP, using p104-nPCR as 'gold standard', in detection of *T. parva* in cattle blood samples

(ii) To determine the levels of concordance of PIM-LAMP assay with the 'gold standard' p104-nPCR to detect *T. parva* in blood samples from cattle.

1.4 Research questions

(i) How accurate is PIM-LAMP in the diagnosis of *T. parva* using cattle blood samples?

(ii) What is the sensitivity of PIM-LAMP in the diagnosis of *T. parva* using cattle blood samples?

(iii) What is the specificity of PIM-LAMP in the diagnosis of *T. parva* using cattle blood samples?

(iv) What is the level of concordance between PIM-LAMP assay and p104-nPCR in the detection of *T. parva* infection in cattle?

1.5 Significance of the study

Two simple, rapid molecular assays, PIM-LAMP and p150-LAMP have been reported for *T. parva* detection in cattle blood. Based on isothermal LAMP technology, they require minimal instructions and are very rapid, with potential for use in modestly equipped laboratories and in resource-poor communities in Africa. However, these assays have not been fully validated for routine use. The main objective of this study was to validate the PIM-LAMP assay and compare its performance with the p104-nested PCR assay.

1.6 Scope

The study utilized 100 archived blood samples from a pool of 1726 cattle blood samples collected from Apac district. The PIM-LAMP assay was the test method evaluated against the p104-nPCR test methods to detect *T. parva* in cattle blood and the diagnostic performance was analyzed by determining the diagnostic accuracy, sensitivity, specificity and the level of concordance of the two methods. Statistical software MedCalc software version 23.0.8 (Ostend, Belgium), a 2 × 2 Table format and Cohen's kappa coefficient were used to evaluate the results.

CHAPTER TWO
LITERATURE REVIEW

2.1 Overview of ticks and tick-borne diseases

Tick and tick-borne diseases (TTBDs) are a group of vectors and diseases that attack cattle, buffalo and other animals. Tick-borne diseases (TBDs) are transmitted by one single species or different species of ticks among animals and humans. The infected animals can either be clinical or healthy carrier animals. Tick-borne diseases are widely spread throughout the world in areas with susceptible hosts but are most prominent in tropical and subtropical regions (Minjauw and Mcleod, 2003), mainly due to the good climate that favours the proliferation of a wide range of tick populations. *Rhipicephalus appendiculatus* is the most economically important vector for *T. parva*, the causative agent of east coast fever (Norval *et al.,* 1992). Important tick-borne diseases in cattle include East Coast fever (theileriosis), heartwater (cowdriosis), redwater (babesiosis) and gall sickness (anaplasmosis) (Byaruhanga *et al.,* 2016). These diseases have contributed to serious economic losses including animal deterioration, loss in fertility, body weight and milk production, leading to an estimated 75.4% loss of cattle productivity (Ocaido *et al.,* 2009). East Coast fever surfaced in the East African region after the introduction of exotic cattle that were susceptible to the disease unlike the local cattle that showed mild illness but recovered (Norval *et al.,* 1992). South Africa has managed to eradicate ECF by managing the spread of ticks, use of immunization and chemotherapy with occasional outbreaks of buffalo-derived *T. parva* (Mbizeni *et al.,* 2013), but theileriosis remains an acute and lethal disease in Burundi, Democratic Republic of Congo, Kenya, Malawi, Mozambique, Rwanda, South Sudan, Tanzania, Zambia, Zimbabwe, Comoro Islands and Uganda (Nene *et al*., 2016).

2.2 *Theileria* species and theileriosis

East Coast fever is caused by parasites of the genus *Theileria* which comprises of tick-transmitted obligate intracellular protozoa of phylum. Apicomplexa, order Piroplasmida and family, Theileriidae (Adl *et al*., 2012). *Theileria* parasites are categorized as either transforming or non-transforming of the host's leukocytes in a way that enables the infected cells to proliferate indefinitely along with the parasites. Among the transforming parasites are, *T. parva, T. serengeti, T. lestoquardi*, and *T. taurotragi* and in the non-transforming are *T. orientalis, T. buffeli, T. mutans,*

T. velifera, and *T. cervi* (Sivakumar *et al*., 2014). *Theileria parva*, the causative agent of ECF, Corridor disease and January disease, occur in Eastern, Central and South Africa whereas *T. annulata* the causative agent of tropical theileriosis occurs around the Mediterranean basin, in the Middle East and in Southern Asia (Norval *et al.,* 2009).

2.3 *Theileria parva* parasite

Theileria parva is a haemoprotozoan parasite, transmitted by the tick vector *R. appendiculatus* and it is the most important tick-borne parasite of cattle in East and Central Africa. It is also the most pathogenic and the most significant economically (Norval *et al.,* 1992). *Theileria parva* belong to the Kingdom: Protista, Subkingdom: Protozoa, Phylum: Apicomplexa, Class: Sporozea, Subclass: Piroplasmia (piroform, round, rod shaped parasite), Order: Piroplasmida, Family: Theileriidae, Genus: *Theileria* and Species: *Theileria parva*. The parasite is known to attack the T and B cells of cattle and wild animals such as the Cape buffalo (Norval *et al.,* 1992).

2.4 *Theileria parva* vectors

Rhipicephalus appendiculatus is a three-host brown ear tick vector that particularly transmits *T. parva*, the causative agent of ECF (Thekisoe *et al.,* 2010). *Rhipicephalus zambeziensis* is also a vector of *T. parva* that leads to a mild and less pathogenic disease of ECF (Odongo *et al.,* 2009). The tick population dynamics is a main factor affecting the efficiency in transmission of TBDs. Climate conditions, vegetation, and host availability are factors that are known to affect distribution of the vector, which in turn determines the distribution of the parasite. These vector ticks are abundant in tropical areas, particularly East Africa (Norval *et al.,* 1992).

2.5 Hosts of *Theileria parva*

The African buffalo (*Syncerus caffer*) is a natural reservoir host of *T. parva* parasite. Other hosts include; cattle (*Bos indicus, Bos taurus*), Waterbuck (*Kobus deffassa*) and the Egyptian buffalo (*Bubalus bubalis*) (Mbassa *et al.,* 1998b). There is evidence of a wildlife-livestock overlap in some South Africa with 100% *T. parva* infection reported in cattle calves and high prevalence in cattle mainly of the buffalo-derived type, indicating a broad sharing of parasites between cattle and buffaloes (Sibeko *et al.,* 2011). Corridor Disease, January Disease and ECF, vary in degree of severity, and this differs among the various cattle breeds (Lawrence *et al.,* 1983). For instance, indigenous breeds of infected animals exhibit endemic stability with mild to subclinical

asymptomatic infection especially in young ones, allowing for the development of immunity after primary infection, while exotic dairy cattle suffer up to 100% mortality if not treated (Sivakumar *et al*., 2014).

2.6 Economic importance of East Coast fever

Theileria parva is the most pathogenic and economically important livestock parasite of the *Theileria* in Eastern, Central and Southern Africa where it causes ECF, Corridor Disease and January Disease respectively (Byarugaba *et al*., 2016). In the 14 affected countries of Southern and Eastern Africa, the disease has led to about one million cattle deaths per year (Patel *et al.,* 2011). In Uganda, ticks and tick-borne diseases (TTBDs) are widespread and are a major threat to cattle, causing substantial mortality and reduced production (Byarugaba *et al*., 2016). Economic losses due to TTBDs to farmers in Uganda were previously estimated at 400 million Uganda shillings annually (Ocaido *et al.,* 2009), mainly through cattle vaccination, tick control by use of acaricides, and anti-theilerial drugs, in addition to losses via cattle weight loss, cattle mortality and low milk reduction.

2.7 Epidemiology of *Theileria parva* and East Coast Fever in Uganda

In Uganda, the epidemiology of *T. parva* is shaped by agro-ecological variation, cattle production systems, vector distribution, and host population structure. Studies conducted across Uganda demonstrate substantial heterogeneity in *T. parva* prevalence (Kabi *et al.,* 2014). In eastern Uganda, a large p104 PCR-based survey of 2,658 cattle in Tororo District reported herd-level prevalence ranging from 0% to 21%, with infections concentrated in the northern and northeastern parts of the district (Muhanguzi *et al.,* 2014). The spatial pattern of infection was strongly associated with the distribution of *R. appendiculatus*, restricted grazing of calves, and selective tick control practices targeting draft animals. At a National scale, a landscape-level assessment of indigenous cattle revealed marked differences across agro-ecological zones (Kabi *et al.,* 2014). Ankole cattle in the southwestern pastoral rangelands, southwestern farmlands, and western highland ranges exhibited significantly higher prevalence of non-clinical *T. parva* infection (36–43%), compared to 17% in East African Shorthern Zebu populations in the northeastern savannah grasslands. Therefore, endemic stability is present in high-prevalence regions, where continuous tick challenge maintains widespread immunity (Kabi *et al.,* 2014).

2.8 Life cycle of *T. parva*

Based on electron microscopy studies, *T. parva* has been observed to undergo sequential development stages in different hosts (Shaw, 1996). *Rhipicephalus appendiculatus*, a three-host tick is the main vector for transmission of *T. parva* species where the process of transmission occurs trans-stadially (Schubert *et al*, 2010). In the cycle, tick larvae or nymphs acquire an infection from piroplasm stage present in red blood cells (RBCs) in the infected cattle while feeding. The infected nymphs and adults then transmit sporozoites, the life-cycle stage which is infective to cattle, during feeding. The sporozoites differentiate into the schizont stage, the schizont stage is multinucleated and is found freely in the host cell cytoplasm surrounded by a basket of host cell microtubules (Shaw, 2003). These infected cells develop cancer like metastatic phenotype and are the primary cause of pathology (Figure 1). Schizonts undergo the process of merogony to produce merozoites, which are released by host cell rapture. Merozoites invade RBCs where they develop into the piroplasm stage free in the RBCs cytoplasm (Shaw and Tilney, 1995). Piroplasms undergo a limited number of cell divisions and cause anaemia due to destruction of infected RBCs (Schubert *et al*, 2010). The piroplasm stage is infective to ticks and differentiation to gametes seems to occur post-ingestion in the tick gut (Schubert *et al*, 2010). Following fusion of macro- and micro-gametes, the resultant zygotes enter cells of the tick gut epithelium and develop into motile kinetes, which are released into the tick haemocel (Figure 1). Sporogony occurs in the E-cells in the type III acinus (Fawcett *et al.,* 1982). *Theileria* sporozoites from the salivary glands are released between 4-8 days' post-attachment by a trickling process (Shaw and Young, 1995) (Figure 1). Approximately 30,000-50,000 sporozoites develop in an infected acinus (Fawcett *et al.,* 1982). Figure 1 is the summary of the life cycle of *T. parva* parasite as illustrated by Nene *et al* (2016).

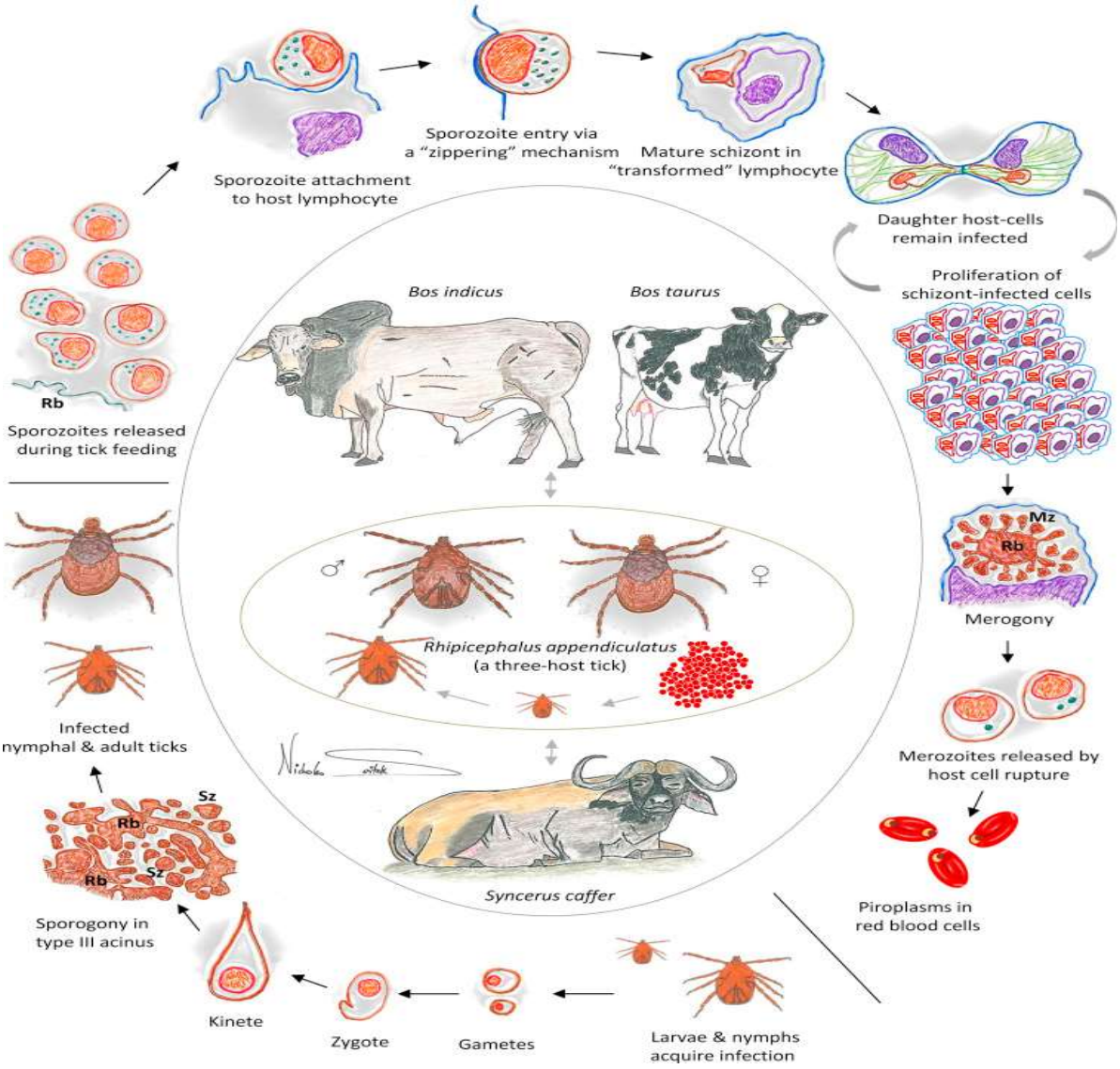

Figure 1: The life cycle of *T. parva* in mammalian host and tick vector (**Nene *et al*., 2016)**

2.9 Pathogenesis of East Coast fever

The infection of cattle with *T. parva* leads to a rapid multiplication of T cells responsible for the pathogenicity (Morrison *et al.,* 1996). *Theileria parva* infection in high doses causes severe lymphohistiocytic vasculitis of the lungs, lymph nodes, spleen, and liver. The lungs contain large numbers of $CD163^{+}$ and $IL\text{-}17^{+}$ macrophages and results in respiratory failure and pulmonary edema during infection, due to the development of pulmonary vasculitis and the induction of a multi-systemic histiocytic response that contribute to the clinical disease (Lawrence *et al.,* 2005).

The incubation period of ECF is about 15 days from the time of attachment of the infected tick. Between 15-25 days of infection, fever remains high, appetite is lost and increasing body weakness, ataxia and frequent recumbence are observed (Lawrence *et al.,* 2005). In the end stages of the disease, the animal expresses the following clinical changes, leukopenia, thrombocytopenia, lymphocytolysis in lymphoid tissues and haemorrhage, leading to death of the animal in coma (Lawrence *et al.,* 2005).

2.10 Transmission of East Coast fever

Transmission of the disease has been aided by cattle in endemic areas that have treatment and/or have spontaneously recovered and have become long-term asymptomatic carriers of *T. parva* schizonts and piroplasms (Kariuki *et al*., 1995). Immunization has also led to the development of a carrier state in cattle, and this has resulted into infection of naïve cattle (Bishop *et al.,* 2002; Oura *et al*., 2007). Carrier status has also been promoted by the *T. parva* strains present in the population and recombinants also attain the same status. However, few strains of this nature are known yet carrier status is quite common with infection-treatment method (ITM) Muguga vaccine treated cattle (Nene *et al.,* 2016). The ability to detect parasites in cattle with the intent of capturing carriers is undermined by the parasite frequencies that seem to fluctuate and fall below detectable levels during sampling time (Geysen, 2000). Studies focusing on DNA markers for parasite strains have shown that *T. parva* carrier animals are a source of parasites which can be transmitted naturally by ticks in the field (Bishop *et al.,* 2002; Oura *et al*., 2007), and yet carrier animals show no signs of clinical disease or loss of productivity (Di Giulio *et al*., 2009).

2.11 Control of East Coast fever

Different methods have been employed to control ECF. Among them is the use of acaricides to control the vector, *R. appendiculatus* (Kivaria*,* 2006), and the use of infection-and-treatment method (ITM) Muguga vaccine to immunize the cattle. Theilericidal compounds are also applied in case of infection with *T. parva,* but they are mostly effective during the early stages of clinical disease and less effective in the late stages of the disease. They are also very expensive to purchase and apply effectively (Sibeko *et al.,* 2010).

2.11.1 Control of Ticks

Tick control in the management of ECF involves the use of physical methods which include the separation of grazing areas to avoid interaction between infected buffalo and cattle, hand picking ticks from cattle, and rearing tick resistant cattle breeds (Muhanguzi *et al.,* 2014). Vudriko *et al* (2018) reported that all cattle farmers in southwestern and northwestern Uganda use acaricides to control ticks. Farmers used chemical acaricides for tick control, predominantly amidine (amitraz) and co-formulated organophosphates and pyrethroids. The spraying method was the most common acaricide application technique, with cattle crush as a common means of physical restraint. Despite the application of acaricides, *R. appediculatus*, the vector for *T. parva* is still proliferating and transmitting theileriosis due to a number of factors including, acaricides resistance due to poor acaricide application methods, the high cost of acaricides, and inappropriate cattle movement across infested areas (Vudriko *et al.,* 2018).

2.11.2 Immunization against ECF

The most effective immunization method against ECF is the Infection and Treatment Method (ITM). The ITM Muguga stabilate is comprised of the Muguga cocktail, the Kiambu 5 and the Serengeti-transformed stock (Mckeever, 2009) and it involves controlled exposure of the cattle to the parasite followed by treatment to induce immunity. The Muguga Cocktail vaccine, developed for ECF, has shown 98% protection against field challenges, making it a cost-effective alternative to frequent acaricide use (Oura *et al.,* 2007). The possibility that the genotypic composition of the stocks that make up the Muguga Cocktail elements could be lost over time is addressed by the rigorous definition of the genotypes present in the Muguga, Kiambu 5 and Serengeti-transformed stocks that comprise the current Muguga Cocktail vaccine stabilate (Mckeever, 2009).

Despite its effectiveness, ECF immunization faces challenges such as limited farmer awareness, high vaccine costs, and the need for proper distribution channels. Additionally, tick resistance to acaricides has become a growing problem, emphasizing the need for sustainable immunization programs.

2.11.3 Chemotherapy

Treatment of ECF with chemotherapeutic agents involves the use of parvaquone, buparvaquone and halofuginone to cure infections and ensures the survival of cattle with clinical *T. parva* or *T.*

annulata infection. However, the effectiveness of these drugs depends on the right application as they become less effective in the advanced stages of the disease when the lymphoid and hematopoietic tissues are adversely destroyed (Sibeko *et al.,* 2010). Early and proper diagnosis of the disease is also challenging, mainly due to the cost of the drugs to the farmers and undermining the application of chemotherapeutics (Nene *et al*., 2016).

2.12 Diagnosis of East Coast fever

2.12.1 Field diagnosis of ECF

Field diagnosis of ECF is normally achieved by observing clinical signs in infected animals. These symptoms normally appear 10 to 25 days after tick attachment and include high fever in the early days of infection (Norval *et al.,* 1992). Other signs are enlarged lymph nodes near the ears and shoulder, depression and lethargy, loss of appetite that leads to rapid weight loss, and pale mucous membrane due to destruction of red blood cells leading to anaemia. Infected cattle have also been presented with diarrhea, laboured breathing, coughing and nasal discharge, cloudness in the eyes in some cases, neurological signs in advanced cases due to parasite invasion of the central nervous system, and sudden death in naïve and untreated animals (Morrison *et al.*, 1996).

2.12.2 Parasitological method for diagnosis of ECF

The parasitological method in *T. parva* detection is microscopy, a longstanding technique that is based on morphological examination of infected cells through blood smears, tissue specimens, and lymph nodes (Mans *et al*., 2015). In cattle, it is used as the first line of differentiation in clinical examination of *Theileria* infection from other infections, and the procedure is based on light microscopy examination of *T. parva* infected blood samples from an infected animal and lymph node needle aspirate smear (Nijhof *et al*, 2005). The Giemsa-stained blood smear microscopy technique is used to examine blood cells. It involves creating a thin film of the specimen (blood) on a clean, dry microscopic glass slide and allowing it to dry in air. The smear is immersed in pure methanol for fixation (2-3 dips) and let to air dry for 30 minutes. The slide is completely covered with a 5% Giemsa stain solution and let to rest for 20-30 minutes. Then the slide is rinsed with tap water and let to dry. The stained blood films are then examined under a microscope using oil immersion lens and observed for the various blood cell types and any abnormalities present (Fahal *et al.*, 2024). The cytoplasm and cytoplasmic granules of blood cells exhibit a red colour while the

nucleus presents a blue-purple colour. Erythrocytes display a pinkish colouration, for the detection of intra-erythrocytic forms of both *Babesia* and *Theileria spp*., piroplasms (Iori *et al*., 2010). Microscopy had a sensitivity of 57% and specificity of 99% while PCR had a sensitivity of 100% and specificity of 100% when testing theileriosis in cattle (Noaman *et al.,* 2014). Microscopy also offered the lowest cost per infection of USD 0.62 as compared to USD 16 for LAMP assay, with the cost driver for LAMP being consumables and personnel (Zelman *et al.,* 2018). Despite the high specificity and low cost, microscopy offers low sensitivity, inability to detect cattle with low parasitemia, carrier status and to distinguish morphologically similar *Theileria* species (Zweygarth *et al*., 1997; Carelli *et al*., 2007).

2.12.3 Serological assays

Serology based methods are more sensitive compared to microscopic examination. They can either be antibody or antigen specific permitting identification of specific parasites in blood smears of infected animals, for example clear distinction between blood borne parasites such as Plasmodium and *Babesia* infections. The serological tests that have been developed to detect *Theileria* infections include Complement fixation assays (CFA), Indirect Fluorescent Antibody test (IFAT) and Competitive inhibition ELISA (Byaruhanga, 2017).

2.12.3.1 Complement fixation Assay

The complement fixation assays (CFA) have been used for diagnosis of *Babesia*, *Toxoplasma*, *Trypanosoma* and *Theileria* parasites. It is based on specific detection of antigens or antibodies in a sample test. A sample of serum is exposed to a particular antigen and complement to determine whether antibodies to that particular antigen are present, the complement is activated by a combination of antigen-antibody complex where lack of antigen specificity leaves the complement free, this method none the less was replaced by the indirect florescent antibody test (Jeffrey *et al.,* 2007).

2.12.3.2 Indirect Fluorescent Antibody test (IFAT)

The Indirect fluorescent antibody test (IFAT) was used to diagnose parasites like *Babesia* and *Theileria* (Darghouth *et al*., 2004; Taha *et al*., 2003). The principle of detection of *T. parva* infection is based on detection of antibodies using cultured *T. parva* schizonts and piroplasm derived from lymphocytes of an infected animal or cell culture as the antigen (Thekisoe *et al.,*

2010). *Theileria parva* antibodies are detectable in serum as early as 30 days and up to 6 months after recovery from the disease, therefore if cell cultures are used, they are able to detect carrier state animals and identify transforming parasites (Zweygarth *et al*., 2009). This procedure is also able to distinguish different species and investigate distinct parasite populations using monoclonal antibodies (Al Hassan *et al*., 2007; Zweygarth *et al*., 2009). However, the procedure is limited by its inability to detect low titre levels and mixed infections such as *T. parva* with *T. taurotragi* (Stewart *et al*, 1996).

2.12.3.3 Competitive inhibition ELISA

Enzyme-Linked Immunosorbent Assays (ELISA) have been used for detection of parasite specific antibodies, antigens and immune complexes (Kachani *et al*., 1992). Recombinant ELISAs have been developed for detection of *Theileria spp.,* using the sporozoite antigen (SPAG- 1) and merozoite surface antigens (Tams-1) for *T. annulata* antigen species (Seitzer *et al*., 2007). Recombinant polymorphic immunodominant molecules (PIM) based ELISA have also been developed and have proved to be better than other ELISAs using other recombinant proteins (p67 and p104 antigens) as well as IFAT because of its higher degree of sensitivity and specificity than the latter (Katende *et al*., 1998). The ELISA method has the advantage of high throughput and being cheap and rapid in the diagnosis of large numbers of samples. The method can also be utilized in areas with limited molecular diagnostics infrastructure. The shortcomings of this method are equally significant, including use of many experimental animals for piroplasm antigen production, batch to batch variation, and protocol standardization (Gubbels, 2000). This challenge has however been subsidized by using recombinant parasite antigens in ELISA such as the recombinant polymorphic immunodorminant molecules (PIM) found in sporozoite and schizont lysates that have been used to develop a serological kit for *T. parva* antigens (Katende *et al.,* 2008). Overall, the major disadvantage of ELISA is that most serological tests rely on presence of specific antibodies which may take days or weeks to develop in an infected animal or may persist for months after infection has been cleared (Katende *et al.,* 2008) leading to misdiagnosis.

2.12.4 Diagnosis of ECF using Molecular Methods

Nucleic acid based diagnostic tests for the detection of *Theileria* spp., are based on the conventional PCR and probing technique (Bishop *et al*., 1992). Amplification of parasite DNA is far more sensitive and specific compared to parasite detection by microscope or IFAT (Sibeko *et*

al., 2008). Molecular methods are also able to simultaneously discriminate parasite species and subspecies which may be present in a blood sample of an infected animal (d'Oliviera *et al*., 1995). Some of these molecular techniques include conventional PCR followed by agarose gel electrophoresis, real time PCR, LAMP, nested PCR and reverse line blot amplification assay (RLB) (Odongo *et al*., 2010; Liu *et al*., 2008; Salih *et al*., 2008).

2.12.4.1 Conventional Polymerase Chain Reaction

Conventional PCR assays using *Theileria parva* Repeat (TpR) gene sequence (Bishop *et al*., 1992; Bishop *et al*., 1995) was developed to detect *T. parva*. However, this assay lacked specificity as they amplified related *Theileria* species. It also failed to amplify *T. parva* infections in field samples because of the high degree of polymorphism in the genes on the TpR locus among the different stocks of *T. parva* (Bishop *et al*., 1995).

2.12.4.2 Reverse Line Blot hybridization assay (RLB)

Probe assays were later developed which make use of *Theileria* specific primers for amplification of small subunits of ribosomal RNA (18S rRNA) genes to detect any *Theileria* species, and subspecies present in the collected sample (Collins *et al*., 2002). The RLB assay is one of them and is a technique where probes are linked in line across the membrane. By adding PCR fragments perpendicular to these lines, positive reactions occur when the fragments and the specific probes in one of the lines hybridize. The RLB has been used for sensitive and simultaneous detection of *Theileria* and *Babesia* species (Altay *et al.,* 2008). The technique involves the simultaneous PCR amplification of related species and making specific PCR reactions for each related species. The species can be identified by a species-specific oligonucleotide probe using line blot apparatus. Specific labelled probes are attached in a line to an activated Biodyne C membrane using a miniblotter. By rotating the membrane of the miniblotter through 90^{o} the biotinylated amplicons are applied to the membrane and across all the probes. During the washing step all non-hybridized material is washed away, and the hybridization is made visible by a peroxidase labeled streptavidin, which interacts with the biotin of PCR product, followed by chemiluminescence detection. The blot with attached probes can be stripped and reused several times (Oura *et al*., 2004). Piennar (2013) describes a RLB method used to detect for *T. parva* in African buffalo and Muhanguzi *et al* (2010) utilized the RLB assay to determine the prevalence and characterization of *Theileria* and *Babesia* species in 363 cattle in Kashaari county, Western Uganda. A comparison

of Reverse Line Blot (RLB) method and β-tubulin targeted nested PCR to detect *Theileria* and *Babesia* piroplasms in cattle found that RLB had a sensitivity of 71.25% and specificity of 57.5% while the sensitivity of the β-tubulin targeted nested PCR was 62.5% and the specificity was 72.5%. The Kappa statistic for level of agreement between the two tests was 0.7984. The RLB assay allowed detection for individual species that simultaneously infected the cattle, whereas β-tubulin targeted nested PCR could not identify species strains. RLB is a relatively simple molecular method that can detect several targets sequences but requires careful probe designs and fully functional molecular biology laboratory (Atuhaire *et al.,* 2013).

2.12.4.3 Real time Polymerase Chain Reaction

Real time PCR (rtPCR) have been employed to detect *T. parva* infections in infected populations of cattle and buffaloes. The technique is more sensitive with almost 100% specificity in carrier animals that contain very low piroplasm concentration (Sibeko *et al*., 2008). Specific primers targeting the 18S ribosomal RNA (rRNA) gene of *T. parva* are designed and extracted DNA is mixed with the primers, nucleotides, and a DNA polymerase enzyme in a PCR reaction mixture. During the PCR process, a fluorescent dye (SYBR Green) binds to the newly synthesized DNA and the fluorescence emitted by the dye as measured by the real time PCR, is directly proportional to the DNA. This allows for the quantification of the parasite's DNA in the sample. The rtPCR machine also generates amplification curves that show the increase in fluorescence over the PCR cycles. The cycle threshold (Ct) value, which is the cycle number at which the fluorescence exceeds a certain threshold, is used to determine the presence and quantity of *T. parva* DNA in the sample. This method has the advantages of high **s**ensitivity and specificity and rapid results**,** detecting low levels of DNA and quantification, making it suitable for identifying carrier animals, and determining parasite load, thereby providing valuable information for disease management (Mamohale *et al.,* 2013).

2.12.4.4 Nested Polymerase Chain Reaction (nPCR)

Nested PCR is another molecular method employed to deal with the setback of unspecific binding of the primers to incorrect regions of the DNA giving rise to unexpected results due to amplifications of non-target regions (Kamolvarin *et al*., 1993). The procedure involves two sets of primers used in two successive runs of polymerase chain reaction. The target DNA undergoes the

first run of PCR using the first set of primers where primer binding gives a product containing the intended sequence. Then the product from the first reaction undergoes a second run using a second set of primers amplifying a secondary target within the first run products, this ensures that the product from the second PCR has little contamination from unwanted products like primer dimers, hairpins and alternative target sequences. Therefore, these non-target sequences are not amplified; hence the method is reported to increase sensitivity and specificity of the reaction producing pure PCR products. However false positives from PCR contaminations or amplifications of nonspecific sequences may still occur (Wilczynski, 2009). Odongo *et al* (2010) described a p104 gene nPCR assay which exhibits enhanced sensitivity for detection of low levels of *T. parva* piroplasms in bovine carrier animals. The assay utilizes the p104 gene of *T. parva* as the basis of detection. The genomic region p104 is widely conserved within *T. parva* strains including stocks from buffaloes that are so genetically diverse and p104 gene is also *T. parva* specific (Skilton *et al*., 2002). However, the relatively complex nature of assays and the need for expensive equipment have made the absorption and utilization of nPCR and other molecular based methods above costly and not widely spread (Mans *et al*., 2015). These challenges have resulted in the need to develop more accommodative molecular techniques for disease diagnosis.

2.12.4.5 Loop-mediated isothermal amplification assay (LAMP)

The LAMP assay is a molecular gene amplification technique that entails high specificity, simplicity, and rapidity (Notomi *et al*., 2004). This procedure allows DNA amplification from a few copies to up to 10^9 copies in less than an hour at a constant temperature using a single DNA polymerase enzyme with high strand displacement activity (Mori *et al.,* 2001). The LAMP primers are designed using primer explorer software available in the Eiken Chemical Co. Ltd. During the reaction, four to six different primers (forward outer, forward inner, reverse outer and reverse inner primers and Loop primers forward and reverse) are specifically designed to recognize six to eight different regions respectively of the target DNA and specifically amplifies the target gene at a constant temperature in strand displacement reaction of DNA polymerase occurring in a single reaction tube and in only two steps, that is, cyclic and non-cyclic amplification (Nagamine *et al*., 2002, Chaouch *et al.,* 2018).

2.12.4.5.1 Design of LAMP assay primers

The design of LAMP primers involves the forward inner primer (FIP) which 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 complementary to the B3c region on the target gene (Figure 2).

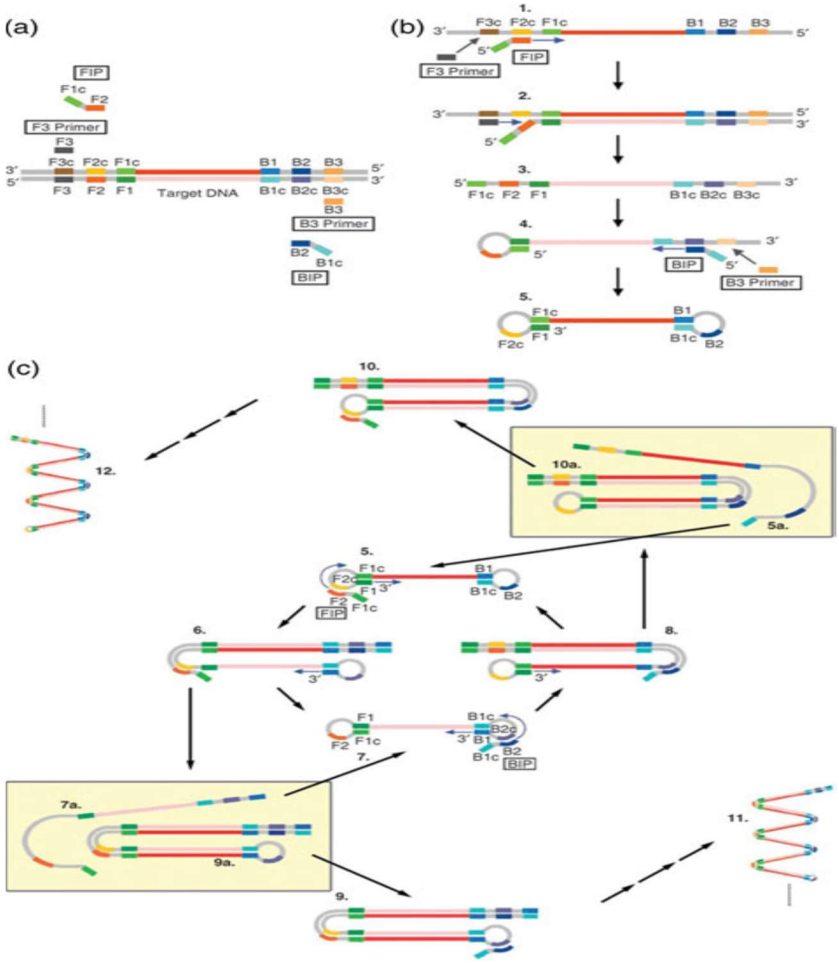

Figure 2: Illustration of LAMP assay reaction (Tomita *et al.*, 2008).

(a) Primer design of the LAMP reaction: Six distinct regions are designated on the target DNA, labeled F3, F2, F1, B1c, B2c, and B3 from the 5′end. As c represents a complementary sequence, the F1c sequence is complementary to the F1 sequence. Two inner primers (FIP and BIP) and outer primers (F3 and B3) are used in the LAMP method. FIP (BIP) is a hybrid primer consisting of the F1c (B1c) sequence and the F2 (B2) sequence.

(b) Starting structure producing step: DNA synthesis initiated from FIP proceeds as follows. The F2 region anneals to the F2c region on the target DNA and initiates the elongation. DNA amplification proceeds with BIP in a similar manner. The F3 primer anneals to the F3c region on the target DNA, and strand displacement DNA synthesis takes place. The DNA strand elongated from FIP is replaced and released. The released single strand forms a loop structure at its 3′ end (structure 3). DNA synthesis proceeds with the single-strand DNA as the template, and BIP and B3 primer, in the same manner as described earlier, to generate structure 5, which possesses the loop structure at both ends (dumbbell-like structure).

(c) Cycling amplification step: Using self-structure as the template, self-primed DNA synthesis is initiated from the 3′ end F1 region, and the elongation starts from FIP annealing to the single strand of the F2c region in the loop structure. Passing through several steps, structure 7 is generated, which is complementary to structure 5, and structure 5 is produced from structure 8 in a reaction similar to that which led from structures 5–7. Structures 9 and 10 are produced from structures 6 and 8, respectively, and more elongated structures (11, 12) are also produced. (Tomita *et al.,* 2008).

2.12.4.5.2 The LAMP assay procedure

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 (Notomi *et al.,* 2000). The process includes a series of stages; first, 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 (Figure 2).

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 and a loop at the opposite end (Figure 2). 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 (Mori *et al.,* 2004 and Notomi *et al.,* 2000). Additionally, two more primers termed loop primers (LoopF and LoopB) are added to make a total of six primers. Loop primers hybridize to the stem loops and accelerate the LAMP reaction thereby significantly reducing the reaction time (Nagamine *et al.,* 2002).

2.12.4.5.3 Detection of LAMP products

The LAMP products are detected by agarose gel electrophoresis stained with ethidium bromide and visualized 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 (Notomi *et al*., 2000). Further, in a positive LAMP reaction magnesium pyrophosphate is formed as a by-product which forms white turbidity in the reaction tube (Mori *et al.,* 2001, Mori *et al.,* 2006). This enables detection by naked eye further saving time of detection. Additionally, fluorescent detection reagent can be added to the LAMP reaction mixture and visualized under UV light (Soliman *et al.,* 2005). 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 (Mori *et al.,* 2004)

2.12.4.5.4 Advantages of using LAMP assay

This method is advantageous as the reaction proceeds at a faster rate (15-60 minutes), than in conventional PCR (2 to 3 hours), a heating block or heated water bath is used instead of a thermocycler, making it simple and inexpensive (Mori *et al*., 2001). Also, since different temperature cycles are not required like in conventional PCR, the reaction processes are simpler and cheaper (Nagamine *et al*., 2002). Furthermore, primers used in LAMP are less sensitive to inhibition as compared to primer activity with *Taq* polymerase in PCR, and this is due to the relative stability of the LAMP reagents at 25ºC and 37ºC (Thekisoe *et al.,* 2010). The LAMP assay therefore holds the potential for field applications in tropical countries where ECF is endemic. This method has been helpful in basic research in medicine, pharmacy, environmental hygiene and point of care testing (Notomi *et al*., 2000). It is currently being used in rapid diagnosis of viral, bacterial, and parasitic diseases as well as surveillance of infectious diseases especially in developing countries which still lack the sophisticated equipment (Tomita *et al*., 2008). Soroka *et al* (2021) describes the general use of LAMP assays for detection of diseases like Foot and Mouth Disease (FMD) in animals, HIV, SAR-COV-2 and malaria in humans, plant viruses and in forensics to

determine the authenticity of plant species, fish species, genetically modified organisms, and origins of meat products.

2.12.4.5.5 *Theileria parva* detection using LAMP assay

The primers of LAMP assay for the detection of *T. parva* have been developed to cover the p67 and p104 *T. parva* gene targeting primers, the heat shock protein 70 (HSP70) gene primer (Thekisoe *et al*., 2007) and the polymorphic immunodominant molecule (PIM) and p150 gene targeting primer (Thekisoe *et al.*, 2010). The primer sets of PIM and p150 *T. parva* gene have been described as the most sensitive and specific of the developed LAMP primers (Thekisoe *et al.,* 2010). Primers from the p67 and p104 genes have shown big differences in the melting temperatures making it difficult to utilize them in LAMP. With regards to conservation, the p150 and PIM genes are polymorphic within their repeat sequence regions between isolates (Toye *et al*., 1995) but the PIM gene is known to contain conserved regions present among many isolates in different countries (Toye *et al*., 1995).

Although LAMP primer sets have been designed from the gene sequence of *T. parva*, using the p150 and PIM gene primers, and are able to amplify isolates from South Africa, Tanzania, Rwanda, Burundi and Uganda, p150 and PIM genes need to be further evaluated for accurate detection of *T. parva* (Thekisoe *et al.,* 2010) on field cattle blood samples to generate supportive data for application of the technique in molecular epidemiology studies.

2.12.4.5.6 Extraction of DNA for LAMP amplification

The conventional DNA extraction methods, such as phenol/chloroform isolation technique or commercial DNA extraction kits, based on spin columns, are laborious, expensive, time-consuming, and require equipment like a high-speed centrifuge (Satya *et al.,* 2013). This limitation hinders their application in field settings where rapid, simple, and equipment-free protocols are essential. Thekisoe *et al* (2010) used the method according to Sambrook and Russell (2001) to extract DNA for LAMP primer specificity and sensitivity tests on blood samples blotted on filter papers (FTA® card, Whatman, UK). The genomic DNA crude extraction from whole blood using an SDS-based lysis protocol adapted from Sambrook and Russell (2001), briefly involves the use of 200 µl of blood mixed with 10% Sodium Dodecyl Sulfate (SDS) solution to make a final concentration of 0.05%, that is then incubated at room temperature for 10 minutes. To another

clean tube, 90 µl of molecular grade water is added followed by 10 µl of lysed blood sample. The mixture is gently mixed and boiled at 90ºC for 10 minutes and then centrifuged. A clear supernatant is used as template DNA for amplification. Chaudhary *et al* (2025) compared four different DNA extraction methods for LAMP amplification, spin column, magnetic bead, Dipstick, and HotSHOT method, for the extraction of DNA from *C. perfringens* and found that spin column and magnetic methods produced higher quality DNA, but required more time, cost, and equipment. But the HotSHOT method provided the fastest, cheapest, and least equipment-dependent extraction, making it the most practical option for field-based detection of *C. perfringens* in poultry samples (Chaudhary *et al*., 2025). The LAMP assay tolerated crude DNA from the HotSHOT method whereas PCR required cleaner DNA and was prone to inhibition (Chaudhary *et al*., 2025).

2.12.4.5.7 Limitations of LAMP assay

The demerit of LAMP is its sensitivity to cross-contamination, due to DNA present in the aerosol. It is therefore recommended that rooms be ventilated, and different samples be analyzed separately. It is also difficult to check the samples for the presence of reaction inhibitors, as this requires two reactions, one to detect the inhibitors and the other to amplify the material. Amplicons cannot also be used for further analyses, such as cloning or sequencing. The target products of the LAMP reaction are short, and as such, any contamination of the sample with exogenous genetic material may impact the outcome. Personnel performing the test should be aware of the risk of contaminating the samples and follow special sterility procedures (Soroka *et al.,* 2021). There has also been a failure to determine the optimal reaction temperatures as has been the case with the p104 gene primers leading to a significant increase in the reaction time (Thekisoe *et al.,* 2010).

2.12.5 Overall changing landscape of ECF diagnosis

The reviewed literature clearly demonstrates an evolution in the diagnostic methods for East Coast Fever (ECF). Traditional Giemsa-stained blood smears are quick and inexpensive; however, they have low sensitivity (57%) and cannot detect carrier states (Noaman *et al.,* 2014). Microscopy is also used as a field standard for clinical cases, but its accuracy depends heavily on the technician's skill and the parasite load. Consequently, this method has gradually been supplemented and replaced by more advanced serological and molecular techniques.

Serological assays, such as ELISA, offer improved sensitivity for detecting antibodies but cannot differentiate between active and past infections (Murunguri *et al.,* 1999). This limitation has resulted in the adoption of molecular methods as the current 'gold standard'. Techniques like Reverse Line Blot (RLB) method (Atuhaire *et al.,* 2013), real-time PCR and the emerging LAMP assay (Thekisoe *et al.,* 2010) provide superior sensitivity and specificity. Moreover, they allow for quantification of parasite load and hold the potential for field-adapted, point-of-care diagnosis.

In this research, we evaluated the diagnostic performance (sensitivity, specificity, and accuracy) of the PIM-LAMP assay compared to the nPCR method, which serves as the standard. Our aim was to determine the suitability of the PIM-LAMP assay for rapid and effective ECF diagnosis and epidemiological surveillance in resource-limited settings. Despite its advantages, challenges related to cost, contamination, and the necessary infrastructure remain.

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study design

This study was conducted in a laboratory setting to evaluate the diagnostic performance of a Loop-Mediated Isothermal Amplification (LAMP) assay for the detection of *Theileria parva.* The study used archived whole blood samples from cattle, originally collected during a previous cross-sectional survey called "Biochemical Phenotyping of Trypanotolerance in Local Cattle," which took place in Apac District, Northern Uganda, from September 2021 to April 2022. The aim of the study was to survey the prevalence of Animal African Trypanosomiasis and potential trypanotolerance in the indigenous cattle in Apac district, Northern Uganda.

3.1.1 Sample selection

From a biobank of 1726 archived samples, a subset of one hundred (100) samples was systematically selected for this analysis. The archived samples were accessed between May 2023 and October 2023 and the selection was based on having sufficient sample volume (greater than 200 µL) and complete metadata. This sample size of 100 was determined to ensure adequate precision for calculating sensitivity and specificity, assuming an expected sensitivity of over 95% and a desired confidence interval width of less than 10%.

3.2 Sample size determination

The sample size was determined using the formula $n = \frac{(z)^2 \times \mathrm{p}(1-\mathrm{p})}{(\mathrm{d})^2}$, sample size estimation in diagnostic accuracy for testing single modality and comparing two diagnostic tasks (Hijian-Tilaki, 2014), where *n* = sample size, Z = 1.96 Z score, a statistic for level of confidence at 95%, p = estimated proportion at 5.3%; prevalence of *T. parva* in cattle in Tororo district (Muhanguzi *et al.,* 2014), and d, the proportion of error at 5%; d = 0.05. Therefore, the estimated sample size was.

$$n = \frac{(1.96)^2 \times 0.053 \times (1-0.053)}{0.05^2} \approx 77 \text{ samples.}$$

The total sample size for this study was adjusted to 100 cattle blood samples, 23 samples above the minimum sample size.

3.3 Cattle blood sample collection and storage

The archived samples were originally collected from indigenous cattle in Apac district, Northern Uganda. The indigenous cattle included, the Lango zebu, falling under the East African zebu, and locally known as "Dyang Lango" or "Lango cattle". The original study screened for trypanozosomes and sampled cattle in 10 sites which are located in the sub-counties of Cegere (6), Ibuje (2), Atik (1), Inomo (2) and Agulu (1). Following appropriate restraint, blood was drawn from the tail coccygeal vein of cattle into a 4ml EDTA vacutainer for downstream analysis. The samples were archived at -20^{o}C in the Molecular Biology Laboratory (MOBILA), COVAB Makerere University. For this study, 100 cattle blood samples were systematically selected from 1726 samples, where the third vacutainer that met the sample selection criteria was selected.

3.4 DNA extraction from cattle blood samples

This study used the Quick-DNA^{TM} microprep Zymo DNA extraction Kit (Murphy Avenue., Irvine, CA, USA) to extract DNA for amplification according to the manufacturer's instructions. Briefly, the genomic lysis buffer (400 µl), and thawed whole blood of cattle (100 µl) were mixed. The mixture was vortexed for 6 seconds and left to stand for 10 minutes at room temperature. The mixture was transferred into a zymo-spin IC column placed into a collection tube and centrifuged at 10,000×g for 1 minute. The filtrate was discarded, and the column was placed back into the same collection tube. Then 200 µl of DNA pre-wash buffer was added to the spin column placed in a collection tube and centrifugation was performed again for 1 minute at 10,000×g. The filtrate was discarded, and the column was placed into a new 500 µl collection tube. Then 500 µl of g-DNA wash buffer was added to the spin column and centrifuged for 1 minute at 10,000×g. The filtrate was discarded. The spin column was then transferred to a clean 1.5 ml Eppendorf tube and 50 µl of DNA elution buffer was added to it. This was incubated for 5 minutes at room temperature and then centrifuged at 10,000×g for 30 seconds to elute the DNA. The eluted DNA was immediately used for nPCR and LAMP or stored at -20^{o}C for future use.

3.5 Screening for *T. parva* positive DNA samples using p104 gene nPCR

To determine the presence of *T. parva* in blood samples targeting the piroplasm single copy gene p104 (Odongo *et al.,* 2010), extracted genomic DNA from cattle whole blood samples together with sterile double distilled water (negative control) and *T. parva* Muguga stock DNA (positive control) were amplified using species specific primer pairs (Table 1) that amplify a 277 bp

fragment using the nested PCR technique (Skilton *et al.,* 2002). For first round PCR, a total volume of 12.5 µl per reaction was prepared and it included 6.25 µl of 2 × master mix per tube (eurofin® genomics, France), 2 pmol/µl of forward primer IL3231 (0.0625 µl) and 2 pmol/µl of reverse primer IL755 (0.0625 µl) (Table 1) plus 3.625 µl sterile double distilled water. Thereafter, 2.5 µl of template (extracted DNA sample) was added. Positive control (*T. parva* Muguga stock DNA) and negative control (double distilled water) were also included. The conditions of PCR were set as follows; initial denaturation at 94 °C for 1 minute followed by 40 cycles of denaturation at 94 °C for 1 minute, annealing at 60 °C for 1 minute and extension at 72 °C for 1 min, plus an additional 9 minutes at 72°C for final extension using Tpersonal Thermal cycler (Biometra®) (Odongo *et al.,* 2010).

For secondary PCR, the reaction mix volume was 12.5 µl consisting of 6.25 µl of 2 × master mix per tube (eurofin® genomics, France), 2 pmol/µl of forward primer IL4243 (0.0625 µl), 2 pmol/µl of reverse primer IL3232 (0.0625 µl) (Table 1), plus 5.125 µl sterile double distilled water and template (1.0 µl of first round PCR products). The reactions were run in a Tpersonal Thermal cycler (Biometra®) at 94^{o}C for 1 minute for initial denaturation, followed by 30 cycles at 94°C for 1 minute, annealing at 55°C for 1 minute and 72 °C for 1 minute, plus an additional 9 minutes at 72°C for final extension (Odongo *et al.,* 2010).

Table 1: The PCR primers for *T. parva* p104 gene amplification (Odongo *et al.*, 2010)

Gene	PCR round	Primer sequence	PCR product (bp)
Piroplasm single copy gene (p104)	1st	5'-TAAGATGCCGACTATTAATGACACC-3' (forward)	496
		5'-ATTTAAGGAACCTGACGTGACTGC-3' (reverse)	
	2nd	5'-GGCCAAGGTCTCCTTCAGATTACG-3' (forward)	277
		5'-TGGGTGTGTTTCCTCGTCATCTGC-3' (reverse)	

3.5.1 Detection of nPCR amplicons using agarose gel electrophoresis

After the nested PCR, the products were analyzed by agarose gel electrophoresis for the expected band size of 277 bp. A volume of 3 µl of ladder, 100 bp DNA marker (N3231S, BioLabs) and amplicons was mixed with 2 µl of 6 × gel loading dye and loaded onto a 2% agarose gel, then the loaded gel was ran in a 1 × Tris-Acetate EDTA (TAE) buffer at 100 volts for 45 minutes. The gel

was observed for band sizes under ultraviolet (UV) light (Figure 3) using gel documentation equipment (Bio-rad Laboratories Inc., USA).

3.6 Loop-mediated isothermal amplification assay for *T. parva* using PIM gene primers

To detect *T. parva* DNA in the selected samples using LAMP assay, extracted genomic DNA from cattle blood was amplified using a set of six primers that targeted the PIM gene (Thekisoe *et al.,* 2010) (Table 2), nuclease-free water (New England BioLabs) was the negative control and *T. parva* Muguga Stock genomic DNA from International Livestock Research Institute (ILRI) was the positive control.

For LAMP assay, 25 µl reaction mixture contained 2.5 µl of LAMP reaction buffer (10× isothermal amplification buffer pack, B0537S, New England BioLabs), 4 µl of 5M Betaine (B0300, Sigma-Aldrich, USA), 3.5 µl of dNTPs (N0447S 10mM Deoxynucleotides solution set), additional 1.5 µl of 100mM $MgSO_4$, (B10035, New England BioLabs Inc.), 0.05 µl of 8000 units/ml *Bst* DNA polymerase (M0538S Bst 2.0 warmstart DNA polymerase, New England BioLabs Inc.), 1.2 µl of primer mix (eurofin® genomics, France) consisting of {0.625 µM forward inner primer (FIP, 0.4 µl) and 0.625 µM backward inner primer (BIP, 0.4 µl), 2.5 µM forward outer primer (F3, 0.1µl) and 2.5 µM backward outer primer (B3, 0.1 µl) and 2.5 µM loop forward primer (LoopF, 0.1 µl) and 2.5 µM loop backward primer (LoopB, 0.1 µl)}, 5 µl of template DNA and 5 µl of genomic *T. parva* Muguga stock DNA extract as positive control, and 7.25 µl of sterile double distilled water. The amplification in the PCR tube was carried out at an isothermal temperature of 65ºC for 45 minutes and stopped by heating the sample at 80ºC for 5 minutes in a Tpersonal Thermocycler (Biometra) (Thekisoe *et al.,* 2010).

Table 2: LAMP primer set for the PIM gene amplification (Thekisoe *et al.,* 2010)

Gene	Name	Primer sequence
PIM	FIP	5'-GGCACAAAGTGCAATCAAAAAGAGAAGAAGTTTCATTTTCGACTGT-3'
	BIP	5'-GCATTGGACTTTTTGCTCATGCTCCTACGGAATAGCCCAAGA-3'
	F3	5'-GTTCTGAGAGCAAAGGCG-3'
	B3	5'-GGCTAACGAGGATAGTACGT-3'
	LF	5'-AGAGATGTGTCAAAAG-3'
	LB	5'-TTCAATGTTGGTGTATTCTTTGCCG-3'

Table 2 shows the LAMP assay primer set designed from the polymorphic immunodominant molecule (PIM) as described by Thekisoe *et al* (2010) where FIP is the forward inner primer, BIP is the backward inner primer, F3 is the forward outer primer, B3 is the backward outer primer, LF is the loop forward primer and LB is the loop backward primer.

3.6.1 Visualization of *T. parva* LAMP products using SYBR green 1 dye

A positive reaction was found by observing the change in colour from orange to green after adding 1 µl of 10% SYBR green 1 dye (Invitrogen Life technologies, Carlsbad, USA) to the LAMP product reaction tubes (25 µl), whereas no colour change was observed for a negative reaction (Figure 4, Panel A).

3.6.2 Detection of LAMP products using agarose gel electrophoresis

To confirm amplification of DNA, agarose gel electrophoresis was used for all the 100 cattle blood samples. A volume of 3 µl of each of the amplicons from the LAMP product reaction tubes was mixed with 2 µl of 6 × gel loading dye and loaded onto a 2% agarose gel, then run in a 1×TAE buffer at 100 volts for 45 minutes. The gel documentation equipment (GelDocGO imaging system, Bio-rad laboratories, USA) was used for imagery of the agarose gel under ultraviolet (UV) light (Figure 4, Panel B), detecting the laddering in the gel.

3.7 Data analysis and interpretation

To determine the diagnostic performance of LAMP assay to detect *T. parva* in cattle blood samples from Apac district, statistical evaluation using MedCalc software version 23.0.8 for windows (MedCalc® Software, Ostend, Belgium) was employed. The following data was generated, the sensitivity, specificity, negative predictive values (NPV), positive predictive values (PPV), and

accuracy (Sadeghi *et al.,* 2021). The level of concordance between LAMP assay and nPCR to detect *T. parva* in cattle blood was determined by Cohen's kappa coefficient (kappa value, κ) where, $\kappa = \{2\times(TP\times TN-FN\times FP)\} \div \{(TP+FP) \times (FP+TN) + (TP+FN) \times (FN+TN)\}$, (Table 3).

Table 3: A 2 × 2 Table comparing nPCR and LAMP assay to detect *T. parva*

		p104-nPCR	
		Present	**absent**
PIM-LAMP	**Test positive**	True positive (TP)	False Positive (FP)
	Test negative	False negative (FN)	True negative (TN)

Table 3 shows the comparison of the detection of *T. parva* in cattle blood samples by LAMP assay and 'gold standard' nPCR. True positives (TP) are cattle with *T. parva* and have tested positive by nPCR, false positives (FP) are cattle with *T. parva* and have tested negative by LAMP assay, false negatives (FN) are cattle without *T. parva* and have tested positive by LAMP assay, and true negatives (TN) are cattle without *T. parva* and have tested negative by nPCR.

3.8 Ethical considerations

This study utilized samples with permission, from the research titled Biochemical phenotyping of Trypanotolerance in the local cattle in Apac district Northern Uganda whose research protocol was reviewed by the School of Veterinary Medicine and Animal Resources Institutional Animal Care and Use Committee (SVAR_IACUC), reference number SVAR_IACUC/64/2020; and cleared by the Uganda National Council of Science and Technology (UNCST), research registration number NS247ES. Community sensitization about the diseases and the role of the screening exercise was first done by the district Veterinary officer with support from the veterinary assistants. Those farmers who agreed to participate in the study were requested to consent before their animals could be screened and samples taken for the study. Approvals included use of the archived whole blood samples for future related work.

CHAPTER FOUR
RESULTS

4.1 Nested PCR assay results for the detection of *T. parva*

The test results for DNA extracts for *T. parva* indicate 21% (21) of blood samples were positive for nPCR using p104 gene primers. Visualization on 2% agarose gel was achieved with ethidium bromide dye under UV light, (Figure 3) and documented with GelDocGO imaging system (Bio-rad laboratories., USA). The amplicons of *T. parva* using nPCR technique were detected at 277 bp on 2% agarose gel (Figure 3).

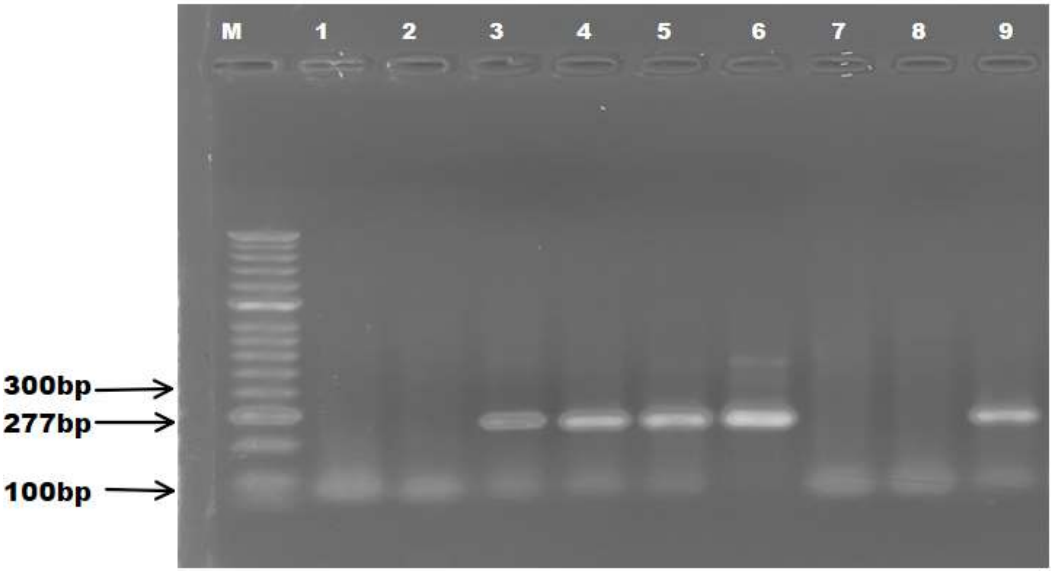

Figure 3: Agarose gel (2%) analysis of p104-nPCR products of *T. parva* genomic DNA.

Amplification of *T. parva* DNA was achieved by targeting the p104 gene in genomic DNA extracted from cattle whole blood samples and positive and negative controls. Lane M: 100 bp DNA marker (N3231S, BioLabs), Lanes 1,2, and 7 are test samples that indicate no amplification, Lanes 3,4,5 and 6 are test samples that indicate amplification for *T. parva* with band size of 277 bp, Lane 8 indicates the negative control, (nuclease-free water, BioLabs), and Lane 9 indicates the positive control, (*T. parva* Muguga stock DNA) which amplified at 277 bp.

4.2 The LAMP assay results for the detection of *T. parva*

The test results for DNA extracts indicate 24% (24) blood samples were positive for *T. parva* using LAMP assay with PIM gene primers. The LAMP assay amplicons were visualized with SYBR

green 1 dye and observed for colour changes. The positive cattle blood samples with *T. parva* parasite in PCR tubes showed green colour (Figure 4, Panel A). The DNA material of *T. parva* in cattle blood samples was confirmed by gel electrophoresis, showing laddering (Figure 4, Panel B).

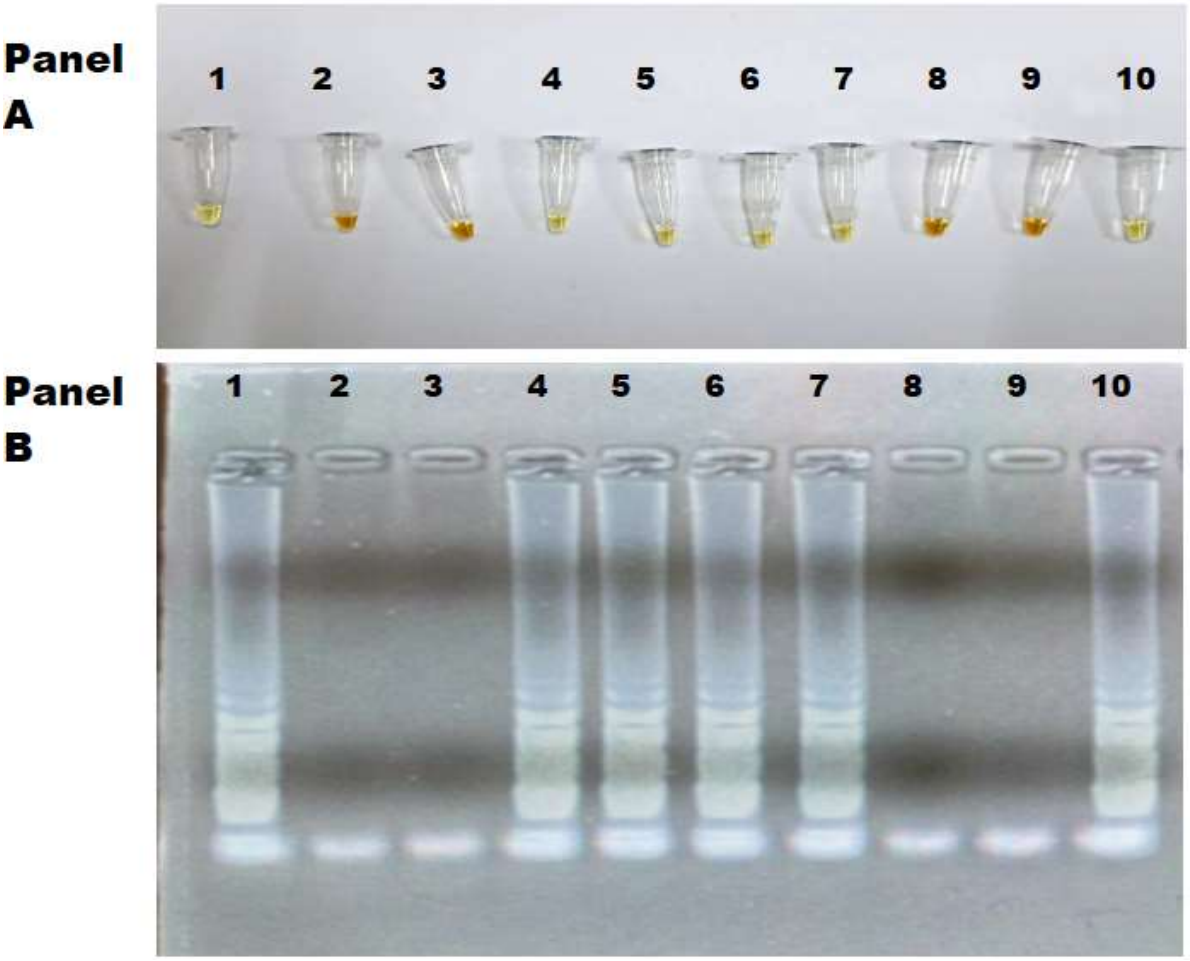

Figure 4: Visualization of PIM-LAMP assay amplicons for detection of *T. parva* DNA. Panel A, using SYBR green 1 dye and Panel B, using 2% gel electrophoresis.

Amplification of *T. parva* DNA by LAMP assay in cattle blood samples targeted the PIM gene and is represented in Panel A; LAMP reaction product tubes with SYBR green 1 dye (Invitrogen Life technologies), tubes 1, 4, 5, 6 and 7 are green test samples, indicating amplification with *T. parva*. Tubes 2, 3, and 8 are orange test samples indicating no amplification. Tube 9 is the negative control (nuclease-free water, BioLabs) without amplification and tube 10 is the positive control (*T. parva* Muguga stock DNA) with amplification. Panel B is a typical 2% agarose gel analysis of the PIM gene LAMP assay products; Lanes 1, 4, 5, 6 and 7: are test samples indicating amplification for *T. parva*, Lanes 2, 3 and 8: are test samples indicating no amplification, Lane 9 is the negative control (nuclease-free water, BioLabs) without amplification, and Lane 10 is the positive control (*T. parva* Muguga stock DNA) with amplicons.

4.3 Diagnostic performance of the LAMP assay to detect *T. parva*

The LAMP assay exhibited a sensitivity of 100%, accurately identifying all 21 samples that tested positive according to the reference standard of nPCR (Table 4). Additionally, the assay demonstrated a specificity of 96.2%, correctly classifying 76 out of 79 samples that were negative by nPCR. However, three samples tested positive with LAMP but negative with nPCR, leading to an overall diagnostic accuracy of 97.0% (Table 4). The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of LAMP assay was evaluated with a P value of 0.001 at 95% confidence interval (Table 4).

Table 4: Diagnostic performance of LAMP assay for detecting *T. parva* in cattle blood samples

Test characteristic	Reading	95% Confidence intervals (CI)
Sensitivity	100.000%	83.890% to 100.000%
Specificity	96.203%	89.301% to 99.210%
Positive predictive value	87.500%	69.762% to 95.503%
Negative predictive value	100.000%	95.262% to 100.000%
Accuracy	97.000%	91.482% to 99.377%

4.4 The level of concordance between LAMP assay and nPCR to detect *T. parva*

The 2 × 2 contingency table presents the LAMP assay with 24 positive cattle blood samples out of 100 tested for *T. parva*, and nPCR with 21 positive samples (Table 5).

Table 5: A reference standard (nPCR) comparison of diagnostic testing with PIM-LAMP assay on DNA samples extracted from cattle blood

		nested PCR		
		Positive	**Negative**	**Total**
LAMP	**Positive**	21 (TP)	3 (FP)	24
	Negative	0 (FN)	76 (TN)	76
	Total	21	79	100

Table 5 shows the comparison of the detection of *T. parva* in cattle blood samples by LAMP assay and 'gold standard' nPCR. Cattle with *T. parva* and tested positive by nPCR were 21, True positives (TP), cattle with *T. parva* and tested negative by LAMP assay were 3, false positives (FP), no cattle without *T. parva*, tested positive by LAMP assay, 0 false negatives (FN), and cattle without *T. parva* and tested negative by nPCR were 76, true negatives (TN).

The level of concordance between PIM-LAMP assay and p104-nPCR to detect *T. parva* in cattle blood samples using Cohen's kappa coefficient (κ) was considered "almost perfect," with $\kappa = \{2\times(21\times76-0\times3)\} \div \{(21+3)\times(3+76)+(21+0)\times(0+76)\} = 0.914$ as computed from Table 5.

CHAPTER FIVE
DISCUSSION

In this study, the diagnostic performance, including sensitivity, specificity, and accuracy of LAMP assay using PIM gene primers (Thekisoe *et al.,* 2010) to detect *T. parva* in one hundred (100) archived cattle blood samples was determined. A diagnostic accuracy of 97.00% (CI: 91.482% to 99.377%), a sensitivity of 100% (CI: 83.890% to 100.00%) and a specificity of 96.2% (CI: 89.301% to 99.210%) were observed. The sensitivity of LAMP (100%) to detect *T. parva* is consistent with Li *et al* (2009), who reported a sensitivity of 100% for the detection of *Salmonella*, Wang *et al* (2012), for the detection of Shinga toxin producing *E. coli* (STEC) in ground beef, Baba *et al* (2014) for the detection of verotoxin-producing bacteria, *Salmonella*, and Shigella in human faeces. The results are also comparable to the sensitivity of 98.5% (Febrer-Sendra *et al*., 2023) for detection of Malaria using field calorimetric LAMP, sensitivity of 97.2% (Menting *et al.,* 2023) for detection of SAR-CoV-2 and 96.7% for the detection of food-borne *Listeria monocytogenes* (Wang *et al.,* 2012). In relation to the sensitivity of 100%, the positive predictive value (PPV) of LAMP was determined as 87.5% (CI: 69.762% to 95.503%), implying that, among the samples identified as positive, 87.5% truly contained the target organism *T. parva.* The PIM-LAMP assay was able to detect *T. parva* in 3 cattle blood samples that tested negative with p104-nPCR, confirming prior reports that LAMP assay is highly sensitive compared to conventional PCR (Wang *et al.,* 2012). This is contrary to the reported specificity of less than 100%, yet LAMP is theoretically more specific than PCR.

The LAMP assay provided a specificity of 96.2% and a negative predictive value (NPV) of 100% (CI: 95.262% to 100.00%), indicating that PIM-LAMP assay can identify all samples that are free of the target gene. Negative predictive value is particularly important in ruling out the presence of the pathogen and minimizing the risk of false negatives. The NPV of 100.00% (CI: 95.262% to 100.00%) observed in this study with a narrow confidence interval suggests that LAMP is a reliable tool for accurately identifying true negative cases, which is crucial for effective disease management and control. In the LAMP method, the target gene is amplified using six pairs of primers improving the reaction specificity and reducing false positive results (Ailenberg *et al.,* 2000). Also with the specificity of 96.2%, though slightly lower than the recommended minimum of 97% required by WHO (Irungu *et al.,* 2021), PIM-LAMP assay is comparable to the specificity

of LAMP assay used to detect Shinga toxin producing *E. coli* (STEC) in ground beef, 100% (Wang *et al* 2012), verotoxin-producing bacteria, *Salmonella*, and Shigella in human faeces, 100% (Baba *et al.,* 2014) and food-borne *Listeria monocytogenes*, 100% (Wang *et al.,* 2012).

The close agreement between the LAMP assay and the 'gold-standard' nPCR (Odongo *et al*., 2010) for detecting *T. parva* in cattle blood was confirmed by a kappa value of 0.91, indicating an 'almost perfect' level of concordance. The high level of concordance beyond chance, indicates that PIM-LAMP assay provides an opportunity for rapid, low cost diagnosis for *T. parva* in cattle.

However, a limitation in the current evaluation is the fact that we did not specifically assess the diagnostic performance of the LAMP assay on cattle blood samples with other confirmed tick-borne diseases like anaplasmosis and babesiosis to validate the specificity of LAMP across different species as part of negative control, though, *T. parva* Muguga stock DNA was considered as a positive control. The study was also limited by the sample size of 100 archived samples. A field based assessment involving fresh samples would allow for the comparison of LAMP assay and nPCR with microscopy, the reference method in detection of *T. parva* in cattle blood samples, and categorizing samples as controls, this would improve the specificity of the LAMP assay for this study.

The LAMP assay technique offers several advantages, including rapid amplification under isothermal conditions, simplicity in primer design, stable reagents, high sensitivity and specificity and the elimination of the need for a thermal cycler. These features make LAMP a practical choice for resource-limited settings where maintaining complex equipment may pose challenges (Criado-Fornelio, 2007). Direct visual identification as demonstrated in the LAMP reactions with SYBR green 1 dye highlights its potential as a robust and efficient screening tool for *T. parva* (Soroka *et al.,* 2021). Other studies have also demonstrated that LAMPamp® fluorescent reagents added to the reaction tube before incubation detected the amplicons by colour change (Thekisoe *et al.,* 2010), a procedure that minimizes environmental DNA contamination of LAMP products during the dye application. Compared to conventional procedures, microscopy, serology, and PCR, LAMP is highly sensitive due to its short-term running period of less than an hour and ability to reduce user-borne errors during the experiment (Mandal *et al.,* 2011).

CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1) The LAMP assay for *T. parva* detection using cattle blood samples evaluated in this present study has a good diagnostic performance (sensitivity; 100.0%, specificity; 96.2% and accuracy; 97%) compared to nPCR.
2) The "almost perfect" level of concordance between PIM-LAMP and p104-nPCR with Kappa value of 0.91 shows that the LAMP assay compares well with the 'gold standard' nPCR in the detection of *T. parva* in cattle blood samples.

6.2 Recommendations

1) Carry out LAMP on a larger sample size with samples classified using a composite reference standard combining microscopy and nPCR.
2) The use of a simple crude extraction method combined with fluorescence dye for visual detection of amplicons of LAMP assay can be employed as a simple and cost effective molecular diagnostic tool for ECF surveillance and prevalence studies in laboratory-limited resource endemic areas.

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APPENDICES

Appendix I: Protocol for detecting *T. parva* using nPCR

To prepare a 12.5 µl reaction tube × 10 master mix

	Item	Unit Quantity (µl)	Quantity (µl) × 10
1st round PCR	Master mix	6.25	62.5
	Molecular grade water	3.625	36.25
	2pmol/µl FP primers (IL3231)	0.0625	0.625
	2pmol/µl BP primers (IL755)	0.0625	0.625
	Template DNA	2.5	2.5 added for each of the 10 PCR tubes
2nd round PCR	**Item**	**Unit Quantity (µl)**	**Quantity (µl) × 10**
	Master mix	6.25	62.5
	Molecular grade water	5.125	51.25
	2pmol/µl FP primers (IL4243)	0.0625	0.625
	2pmol/µl BP primers (IL3232)	0.0625	0.625
	Template DNA (1st round amplicons)	1.0	1.0 added for each of the 10 PCR tubes

1st round PCR

A 100 µl master mix (without template DNA) was prepared and dispersed into PCR tubes in volumes of 10 µl each, and then 2.5 µl of template DNA (including DNA ladder, positive control and negative control) was added to each of the PCR tubes to make a final volume of 12.5 µl reaction tube. The PCR tubes mixture was incubated at 94°C for 1 minute for initial denaturation followed by 40 cycles of 94 °C for 1 minute, annealing at 60°C for 1 minute and extension at 72 °C for 1 min, plus an additional 9 min at 72°C for final extension using Tpersonal Thermal cycler (Biometra®)

2nd round PCR

A 100 µl master mix with 2nd round PCR primers (without template DNA) was prepared and dispersed into PCR tubes in volumes of 10 µl each, and then 1.0 µl of template DNA (amplicons from 1st round PCR) was added to each of the PCR tubes to make a final volume of 12.5 µl reaction

tube. The PCR tube mixtures were incubated in a Tpersonal Thermal cycler (Biometra®) at 94ºC for 1 minute for initial denaturation, followed by 30 cycles at 94 °C for 1 minute, annealing at 55°C for 1 minute and 72 °C for 1 minute, plus an additional 9 minutes at 72°C for final extension.

Gel electrophoresis and photography using image documentation equipment was used to visualize the DNA amplicons on 2% agarose gel under UV light.

Appendix II: Protocol for detecting *T. parva* using LAMP assay

To prepare a 25µl reaction tube × 10 master mix

Item	**Unit Quantity (µl)**	**Quantity (µl) × 10**
Isothermal Buffer	2.5	25.0
Molecular grade water	7.25	72.5
Bst enzyme	0.05	0.5
100µM inner primers	0.4× 2	4.0 × 2
100µM outer primers	0.1 × 2	1.0 × 2
100µM Loop primers	0.1 × 2	1.0 × 2
100mM magnesium sulphate	1.5	15.0
5M Betaine	4.0	40.0
100mM dNTPs	3.5	35.0
Template DNA	5	5 added for each of the 10 PCR tubes

A 200 µl master mix (without the template DNA) was prepared and dispersed into 10 PCR tubes in volumes of 20 µl each, and then 5 µl of Template DNA (including positive control and negative control) was added to each of the PCR tubes to make a final volume of 25 µl reaction tube. The PCR tubes mixture was incubated at 65ºC for 45 minutes and the reaction stopped at 80ºC for 5 minutes.

Visualization of LAMP assay amplicons was by gel electrophoresis, SYBR green 1 dye and photography using image documentation equipment.

Appendix III: Protocols for visualization of DNA amplicons

i) Visualization using SYBR green 1 dye for LAMP amplicons

To 9 µl of sterile double distilled water, add 1µl of SYBR green 1 stock solution to make 10^{-1} dilution for 10 PCR reaction tubes, protect from sunlight. To the LAMP products in PCR tubes, add 1µl of 10% SYBR green 1 dye, gently tape the PCR tube and observe for colour change. The green colour indicates samples with amplified DNA (positive for *T. parva*) and the orange colour in PCR tubes indicates no amplification.

ii) Visualization using Gel electrophoresis

The DNA amplicons were applied on 2% agarose gel. The electrophoresis buffer was prepared by adding 20ml of Tris acetate EDTA (50×TAE) buffer to 980ml of distilled water, to make a 1×TAE buffer solution and stored. The agarose gel mold was prepared by combining 20 ml distilled water with 0.4g of agarose. The agarose mixture was heated on the hot plate until all the agarose had completely dissolved. The agarose solution was allowed to cool to about 55ºC and then poured into a gel mold, in which the comb is inserted. The gel was allowed to solidify at room temperature for 20 minutes, thereafter, the comb was removed. The gel was placed in an electrophoresis chamber with enough 1×TAE buffer to cover the gel. The gel was placed with the comb in the negative side of the electrode. Then 3 µl of amplified DNA elute was mixed with a 2 µl of 6 × ethidium bromide dye, prepared by adding 25 µl of ethidium bromide stock to 1L of 1×TAE buffer and stored in an amber bottle to protect it from direct sunlight. Then a volume of 2 µl of the DNA elute and ethidium bromide was placed into comb well, and the chamber was covered. The electrophoresis chamber was powered by 100V for 45 minutes and stopped. The DNA amplification was observed in visual GelDocGo imaging system (Bio-rad laboratories Inc., USA), from which the DNA bands for nPCR and laddering for LAMP assay were observed.

iii) composition of Tris-Acetate EDTA (50 × TAE stock solution) buffer

- Tris hydroxymethyl amine methane base: 242 g
- Glacial acetic acid: 57.1 ml
- Ethylene diamine tetra acetic acid (EDTA): 0.5M EDTA
- Distilled water: Adjust with distilled water to 1 Litre