Use of polymerase chain reaction (nested) and loop mediated isothermal amplification (wet and lyophilized) for the detection of *Toxoplasma gondii* in clinical samples

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

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DEDICATION

To my father Mr. Albert Thiong'o, late mother Mrs. Grace Njeri, My wife Margaret Wakonyu and my children Victor Thiong'o, Purity Njeri and Pauline Gakenia. All have been a great source of encouragement and inspiration in my pursuit for career achievement.

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LIST OF ACRONYMS

AIDS	Acquired immunodeficiency syndrome
B3	Backward
BIP	Backward inner primer
Bst	*Bacillus stearothermophilus*
CNS	Central nervous system
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DT	Dye test
dNTPs	Deoxynucleoside tri-phosphate
ELISA	Enzyme-linked immunosorbent assay
F3	Forward
FIP	Forward inner primer
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
IFAT	Indirect fluorescent antibody test
IHAT	Indirect human agglutination test
IgA	Immunoglobulin A type

IgE	Immunoglobulin E type
IgG	Immunoglobulin G type
IgM	Immunoglobulin M type
ITS	Internal transcribed spacer
KCl	Potassium chloride
LB	Loop backward
LF	Loop forward
LCR	Ligase chain reaction
LAMP	Loop-Mediated Isothermal Amplification
mBar	Millibar
MgSO4	Magnesium sulphate
mL	Millilitre
mM	Millimolar
NASBA	Nucleic acid sequence-based amplification
NATS	Nucleic acid amplification tests
NC	Negative control
NHPs	Non human primates

PC	Positive control
PCR	Polymerase chain reaction
nPCR	Nested polymerase chain reaction
pM	PicoMolar
RPM	Rounds per minute
RNA	Ribonucleic acid
RT- LAMP	Reverse transcriptase Loop-Mediated Isothermal Amplification
SDA	Strand displacement amplification
WHO	World health Organization
^{o}C	Degrees centigrade
µl	Microliter

ABSTRACT

Toxoplasmosis is a disease in humans caused by a protozoan parasite known as *Toxoplasma gondii*. The widely used methods of diagnosis of *T. gondii* are mainly serological but have low sensitivity and are labor intensive. There is need for alternative diagnostic tests that are more sensitive and can be used when specific antibody titers are below detectable threshold levels. The amplification of repetitive 529 bp loci of *T. gondii* has been reported to be sensitive and specific and was amplified in this study. The objective of the study was to compare the performance of nested polymerase chain reaction (nPCR) with loop mediated isothermal amplification (LAMP) (wet and dry). A total of 87 human blood samples collected from the slaughter house workers between March 2013 and June 2013, and another 87 baboon blood samples collected between 2006 and 2011 were studied. The DNA extracted from the samples was analyzed using nested PCR and LAMP (wet and dry) based on the same target. In overall, 39.1% of the slaughter house workers and 33.3% of the baboons tested positive by nested PCR. *T. gondii* was detected in 33.3% and 42.5% of the slaughter house workers by wet LAMP and dry LAMP respectively. There was no significant difference in the performance between nested PCR and LAMP (wet and dry) and also between the wet LAMP and dry LAMP (p value >0.05). This study reports high prevalences of *T. gondii* infection in high risk group of workers and baboons in Thika District. This study recommends the need for public health awareness through education in the control of *T. gondii* in Thika District. Moreover, the baboon (*P. anubis*) can be used as an experimental model in the investigation of transmission, diagnosis and treatment of *T. gondii* infection. The study further demonstrates that lyophilized LAMP may be used in the field set up for the detection of *T. gondii*. However, further evaluation on reagent stability after storage at different temperature and duration should be investigated.

CHAPTER 1

1.0 INTRODUCTION

1.1 Background

Toxoplasma gondii is a protozoan parasite causing toxoplasmosis in warm blooded vertebrates (Tenter *et al*., 2000). The parasite is distributed globally and infects a wide range of warm blooded animals including humans (Montoya, 2002; Khan *et al*., 2006). In humans, the parasite has been reported to cause congenital toxoplasmosis in developing fetus and is life threatening to immunocompromised patients, such as those with advanced HIV infection or those recovering from organ transplantation (Dubey & Jones, 2008). In immunocompetent humans, toxoplasmosis mostly remains subclinical. The clinical manifestations when present are usually non-specific and mimic other infections which complicate definitive clinical diagnosis (Hill and Dubey, 2002; Kompalic-Cristo *et al*., 2004).

Diagnosis of toxoplasmosis can be accomplished by immunological testing, histological identification technique, parasite isolation by tissue culture, parasite DNA detection by polymerase chain reaction (PCR) or by a combination of these techniques. Cerebral toxoplasmosis can be diagnosed by computerized tomography and magnetic resonance imaging (Hill and Dubey, 2002; Markus, 2003; Sukthana, 2006).

Diagnosis of *T. gondii* infection in patients is based on the detection of parasite specific immunoglobulin M (IgM) in serum of patients by Indirect Fluorescent Antibody Test (IFAT) and Enzyme-Linked Immunosorbent Assay (ELISA). The antibody detection is labor intensive, time-consuming, and expensive. In addition, the antigens used in the antibody detection assays contain extraparasitic materials which result in inter-assay variability (Montoya, 2002). In this regard,

there is need for more sensitive tests that can be applied in the field such as loop-mediated isothermal amplification (LAMP) of DNA (Notomi *et al*., 2000).

1.2 Statement of the problem

The current global prevalence of *T. gondii* in humans has been reported to be 30% (Montoya & Remington, 2008; Zemene *et al*., 2012). However, in Kenya, studies on toxoplasmosis in human are limited and the few available reports indicate high sero-prevalence of the disease (Bowry *et al*., 1986). A recent study in Thika District has established the occurrence of potential risk factors such as the presence of reared and stray cats which contribute to the transmission of toxoplasmosis (Ogendi *et al*., 2013).

The current diagnostic methods for toxoplasmosis are mainly serologic and based on detection of anti *T. gondii* specific antibodies or the antigens. These serologic tests include enzyme-linked immunosorbent assay (ELISA), indirect fluorescence antibody test (IFAT), indirect human agglutination test (IHAT), the dye test (DT) and immunoblotting (Wastling *et al*., 1994; Kimbita *et al*., 2001; Montoya, 2002). Serologic assays have been associated with many limitations such as long procedures that lead to delayed results, high cost of the test kits and low sensitivity all which make the tests unreliable for diagnosis of toxoplasmosis (Montoya, 2002).

1.3 Justification of the study

The use of nested PCR (nPCR) has been shown to be more sensitive and specific for detection of *T. gondii* infection than the serological tests (El-Madawy& Metawea, 2013). The target genes that have been used for detection of the infection include 18S ribosomal DNA, B1 multi-copy element, SAG 1, SAG 2 and 529bp multi-copy element. Previous studies have shown that 200-300 fold 529bp repeat element is a reliable target for the detection of *T. gondii* DNA by nested

PCR (Reischl *et al*., 2003). However, the application of PCR is still limited to biomedical research due to high cost of the thermo-cyclers and the long reaction times (Guy *et al*,. 1996).

Reports by several investigators have shown amplification of DNA by LAMP methods to be reliable for field application. Tests based on LAMP have been developed for *Plasmodium* species (Poon *et al*., 2006), *Trypanosoma* species (Kuboki *et al*., 2003; Thekisoe *et al*., 2005) and *Babesia* species (Ikadai *et al.,* 2004). This technique leads to reduction in cost and time (Iwasaki *et al*., 2003; Poon *et al*., 2006).

Previous studies have shown that LAMP based on 200-300 fold 529 bp repeat fragment in *T. gondii* genome has high specificity and sensitivity compared with nested PCR (Zhang *et al*., 2009; Kong *et al*., 2012). In this study, the suitability of 529bp repeat fragment in nested PCR and LAMP was assessed using sera from high risk group of workers and from baboons (*P. anubis*) which scavenge food in Thika District. The performance of dry reagent for LAMP reaction was further determined.

1.4. Research objectives

1.4.1. General objective

To detect *T. gondii* DNA in sera by use of nested polymerase chain reaction and wet loop mediated isothermal amplification in comparison with dry LAMP.

1.4.2. Specific objectives

1. To determine the occurrence of *T. gondii* DNA in human and baboon serum samples in Thika District using nested PCR

2. To determine the performance of wet LAMP reagent in *T. gondii* DNA detection

3. To develop dry LAMP reagent for *T. gondii* DNA detection

4. To compare performance of dry LAMP reagents with nested PCR and wet LAMP for the detection of *T. gondii* DNA

CHAPTER 2

2.0 LITERATURE REVIEW

2.1. Introduction

Toxoplasma gondii is a protozoan parasite that belongs to phylum Apicomplexa and subclass Coccidia (Dubey *et al*, 2004; Kopecna *et al*., 2006). The parasite is present in about 30 percent of the global human population spread in all continents (Abu-Madi *et al*., 2008; Nissapatorn, 2008; Dubey, 2004). Numerous social economic losses have been associated with the parasite such as deaths, morbidity and permanent deformities in human and animal (CDC, 2000) hosts.

2.2. Geographic distribution

In human, *T. gondii* infection is widespread in all the continents. Serologic surveys have shown that the prevalence of infection vary from place to place depending on the sampling techniques used. For instance in the United Kingdom and the United States, 10-40% of the people are infected, while in continental Europe and Central and South America, the infection ranges from 50-80%(Jones *et al*., 2007). In Africa, *T. gondii* seropositivity has been reported in several countries. For instance in Mali a sero-prevalence of 27% has been reported (Ouologuem *et al*., 2013). In Tanzania and Ethiopia sero-prevalences of 30.9% and 83.6% respectively have been reported in pregnant women (Mwambe *et al*., 2013; Zemene *et al*., 2012). In Kenya, sero-prevances of up to 60% have also been reported in preschool children (Bowry *et al*., 1986). Broader global prevalences of *T. gondii* infection in human are provided in Fig. 2.1.

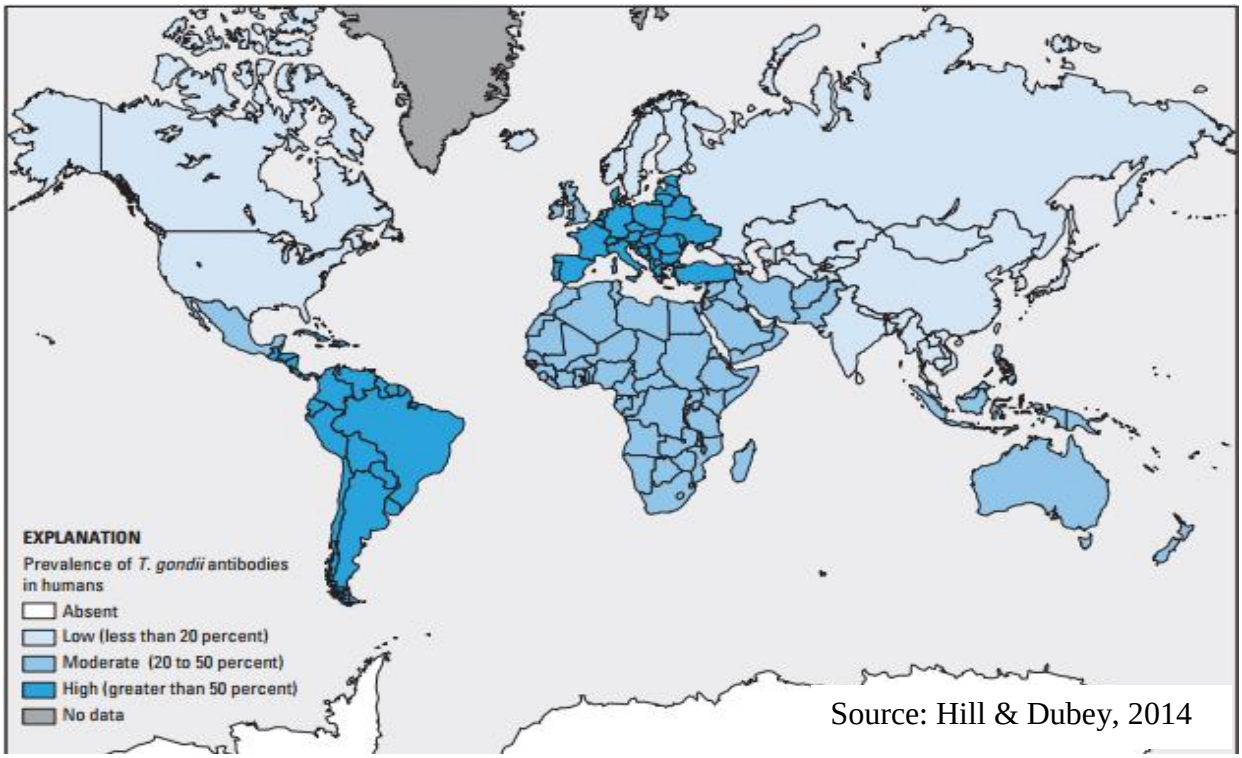


Fig. 2.1. Geographic distribution of the prevalence of *T. gondii* antibodies in humans

2.3. *T. gondii* clonal lines

Different strains of *T. gondii* are distributed in three clonal lines. These strains of *T. gondii* occur in different geographical locations within different communities. The molecular genotype of the African strains is not well studied unlike the strains found in South America, Europe and Asia. Genotypic characterization of *T. gondii* has shown that roughly 90% of the analyzed isolates can be clonally grouped into three lineage-types I, II and III. The clonal lineages have varied degrees of pathogenicity and have been reported in Europe and North America. The RH strain, named after the name initials of a boy from whom it was first isolated (Sudan *et al* ., 2014), represents type I and has been reported as the most virulent in murine infections, compared to type II and III strains which are relatively less virulent (Peyron *et al*., 2006). In the mouse model, acute virulence of these strains has been well characterized with type I generally causing highest

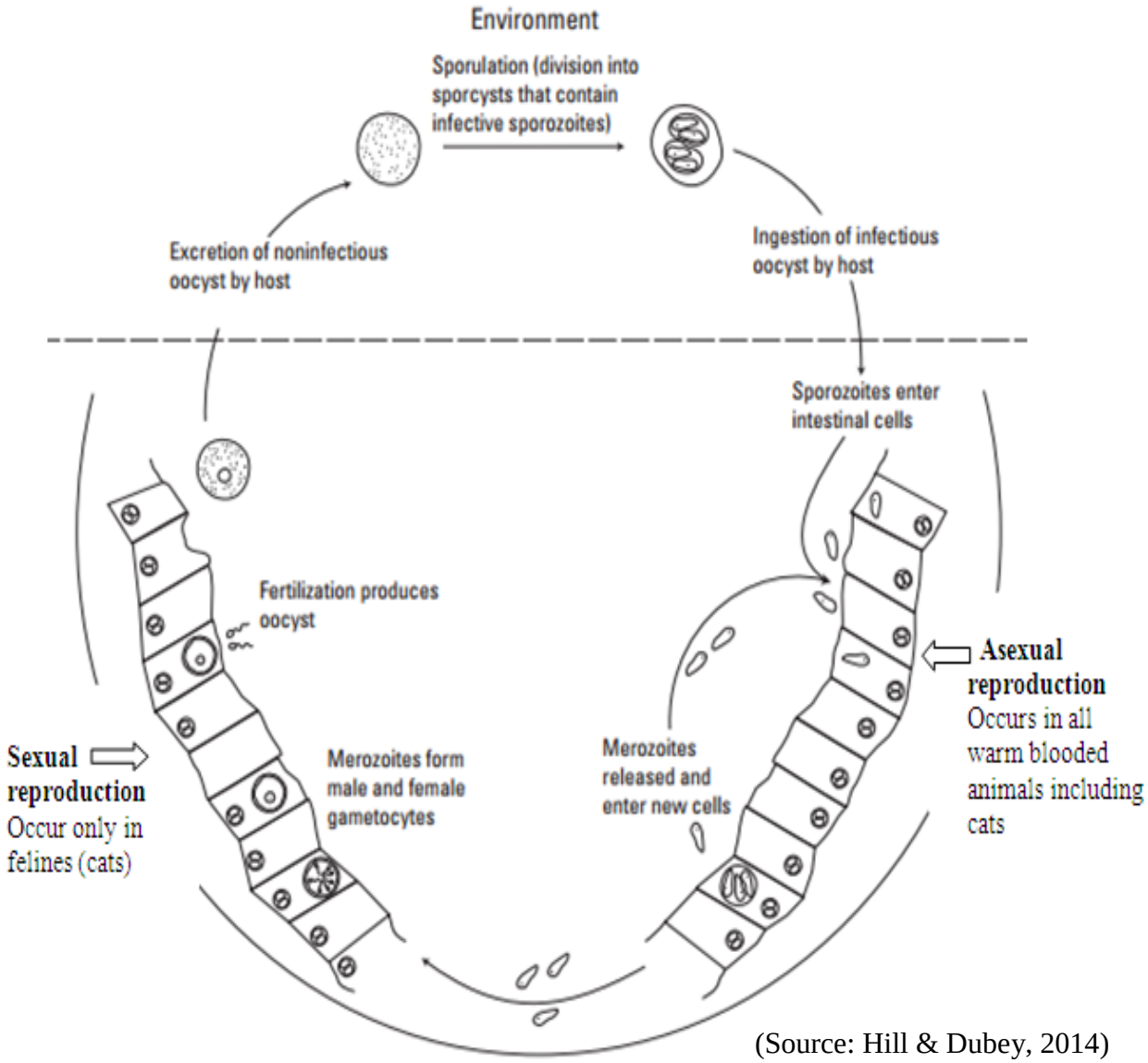
mortality in a few days following intraperitoneal challenge with a single tachyzoite. Lethal doses with types II and III have been reported to involve10 fold of the organisms following the intraperitoneal infection of the parasites. Moreover, a challenge of BALB/c mice with types II and III led to a chronic infection (Sibley *et al*., 2002).

A previous study on the prevalence of *T. gondii* in indigenous free-range chicken (*Gallus domesticus*) was conducted in Africa specifically in Democratic Republic of Congo, Burkina Faso, Mali and Kenya. The study found out that all the three clonal lineages also occur in African continent. In Kenya, where 4 in30 chicken tested sero-positive, only type II strain was isolated from the brain of one of the sero-positive chicken (Dubey *et al*., 2005). In Ghana, a strain with combination of lineages I and III has been isolated from a HIV patient (Genot *et al*., 2007). However the genotype of the African *T. gondii* strains is not well understood.

2.4. Life cycle

The domestic cats and other felid animals are mainly the definitive hosts to *T. gondii* and become infected after ingestion of infected meat or prey such as the rats. The sexual and asexual cycles of *T. gondii* parasite occur in the cat's intestinal epithelial cells. Other warm blooded animals such as human and birds serve as intermediate hosts and can only harbor the asexual stages of the parasite (Dubey, 2008; Dubey, 2004). The infected cats shed millions of oocysts together with feces which contaminate the environment (Dubey, 2004). The oocysts take between 1 and 5 days to transform into infective forms in the presence of adequate moisture and temperature. The infective oocysts mechanically gain access into the intermediate host through contamination of ingested materials such as food. The infective oocysts get swallowed and enter the digestive tract of the host (human). The parasites actively penetrate the intestinal epithelium and enter the blood stream to invade the nucleated cells. In the parasitophorous vacuole, the parasites get

transformed into tachyzoites and divide asexually by endodyogeny process to increase in numbers (Abu-Dalbou *et al*., 2010). The vacuoles rupture and release the tachyzoites which infect new cells (Lingelbach & Joiner, 1998). In the fourth day following the infection, the tachyzoites may appear in the circulation and mesenteric lymph nodes (Dubey, 2004). The rate of multiplication is dependent on the host's immunologic defense mechanism. Immunocompetent hosts are able to subdue the multiplication of the parasite and tachyzoites invade the muscular and neural tissues such as the skeletal and cardiac muscles, eyes and brain. In the tissue cells, the tachyzoites transform into bradyzoites which persist for life in the hosts as tissue cyst and get reactivated into infective tachyzoites in circumstances of host immunosuppression (Abu-Dalbou *et al*., 2010).



(Source: Hill & Dubey, 2014)

Fig. 2.2. The life cycle of *T. gondii* showing the sexual and the asexual phases of the parasite

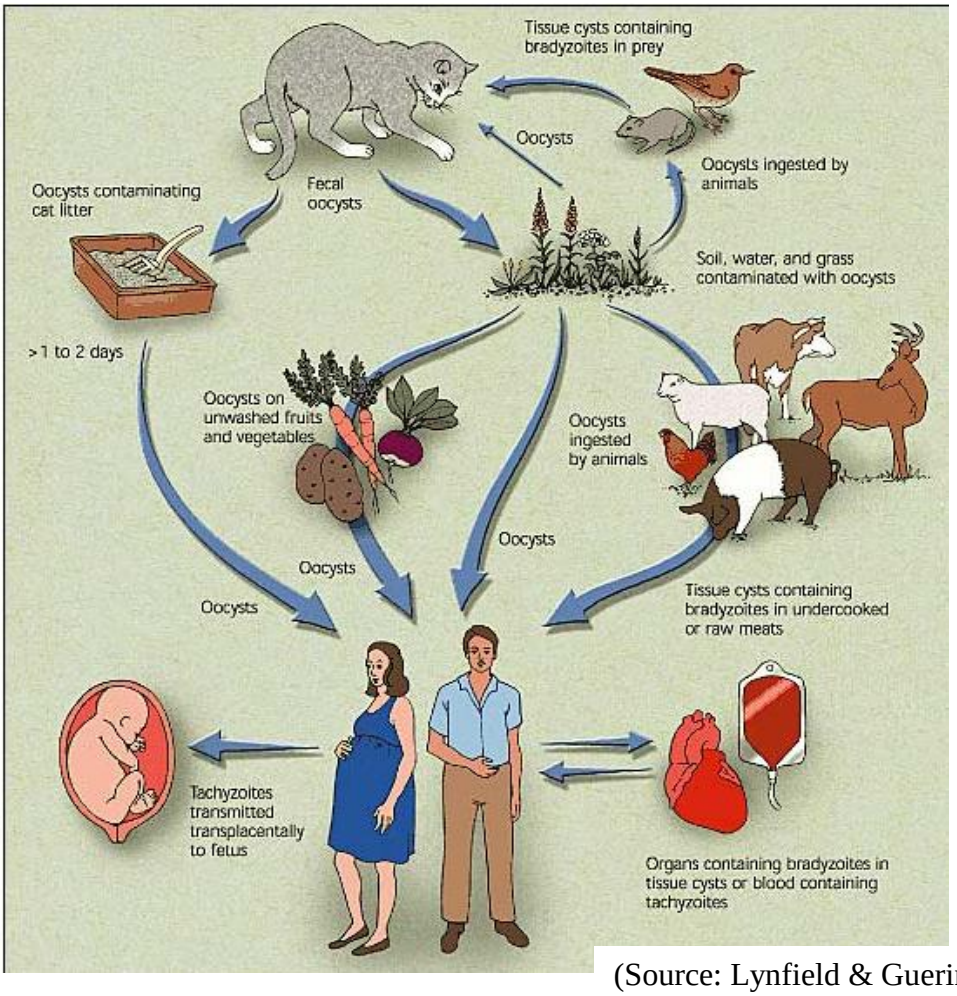
2.5. Disease

In humans, the parasite causes toxoplasmosis and the infection may be subclinical or may clinically manifest with mild non-specific symptoms in immunocompetent humans. However in immunosuppressed patients such as the HIV patients, *T. gondii* infection may lead to cerebral toxoplasmosis which is life threatening. Toxoplasmosis that involves the central nervous system

is associated with multi-focal lesions and diffuse encephalitis. Clinically, the immunocompromised patients with *T. gondii* central nervous system (CNS) involvement normally present sub-acute or acute manifestations depending on the number of the lesions present (Manzardo *et al*., 2005). A recent report has hypothesized that brain cancer could be triggered by *T. gondii* (Thirugnanam *et al*., 2013). In pregnancy, the infection has serious consequences such as abortion, still birth, enlargement or reduction in child head size, seizures and vision loss (Couvreur, 2001; Montoya, 2002; Ghazaei, 2006). Ocular toxoplasmosis may occur as a result of congenital transmission or reactivation of the tissue parasites and may lead to toxoplasmic retinochoroiditis and eventually retinal lesions. A recent report has shown that up to 82% of individuals with congenitally acquired toxoplasmosis and not treated at infant stage may develop retinal lesions (Kianersi *et al*., 2012). Moreover, other complications involving the heart, lung and skin have been associated with toxoplasmosis (Weiss & Kim, 2007).

2.6. Transmission

Humans generally become infected by ingestion of raw or insufficiently cooked meat and pork containing viable bradyzoites, or by ingestion of infective oocysts that contaminate fruits and vegetables. Pregnant women infected for the first time or the immunosuppressed pregnant women pose high risk of spread to fetus leading to congenital *T. gondii* infection in neonates. The reactivation of the dormant bradyzoites in the tissue cysts into infective tachyzoites in immunosuppressed patients is a common occurrence (Lindström *et al*., 2006). The parasite may also be spread by organ transplantation and blood transfusion (CDC, 2000; Vaz *et al*., 2010).



(Source: Lynfield & Guerina, 1997)

Fig. 2.3. Routes of *T. gondii* transmission

2.7. Diagnosis

The effective control and management of toxoplasmosis is central to accurate diagnosis and proper treatment of the patients since no vaccines are available currently. The detection methods range from those which directly target the whole parasite in human samples to those that target the parasite indirectly by detection of anti *T. gondii* specific antibodies. A range of test kits have been developed and are capable of producing quantitative and semi-quantitative results (Joynson & Guy, 2001).

2.7.1. Serological methods

The detection of anti *T. gondii* specific antibody is considered the first line in diagnosis of acute toxoplasmosis (Beghetto *et al*., 2006).Different serological based methods for the detection of *T. gondii* have been developed and are widely used. The methods are based on the detection of different anti-*T. gondii* specific antibodies and the methods commonly used are prone to misinterpretation of results (Montoya & Remington, 1995). The persistence of specific anti-*T. gondii* antibodies may erroneously be reported as a positive test even though there may be no parasites in the circulation.

2.7.1.1. Modified agglutination test

The modified agglutination test (MAT) is mainly used to detect anti-*T. gondii* IgG antibodies (Remington *et al*., 2001). The IgG antibodies appear in the circulation about 1 to 2 weeks and reach the peak at about 1 to 2 months. The levels of the IgG antibodies then drop at various rates or may persist indefinitely. The MAT may not be able to distinguish between a recent infection and that acquired in the distant past. In such a case, a different parallel test would be required (Montoya, 2002). Moreover, MAT kits are designed specifically for different animal species and therefore the kits may not be readily available, for instance, the MAT kits for the detection of *T. gondii* in humans are not currently available.

2.7.1.2. Indirect hemagglutination test

The detection of antibodies by IHAT method only indicates a past infection with *T. gondii* and not the current and usually high titers of specific antibodies are required for detection (Montoya, 2002). The protocol requires dilution of serum samples for instance starting from 1:80 then 1:160, followed by titration with soluble antigens to determine the highest titre upon which a decision is made as to whether or not the serum is truly sero-positive (Alazemi, 2014). The

agglutination occurring at titre of 1:80 is regarded sero-positive. However, the accuracy and sensitivity of the method is the main challenge (Kaye, 2011).

2.7.1.3. Indirect fluorescent antibody test

The indirect fluorescence antibody tests (IFAT) are designed as kits that measure anti-*T. gondii* IgG antibodies or anti-*T. gondii* IgM antibodies (Remington *et al*., 2001). The IFAT tests have the limitation of low specificity and are easily misinterpreted. Determination of exposure to *T. gondii* by IFAT requires a combination of detection methods (Montoya & Remington, 1995).

2.7.1.4. Immunosorbent agglutination assay

In human, the presence of specific IgM antibodies can be determined by the use of immunosorbent agglutination assay (ISAGA). In this technique, the utilization of monoclonal antibody enables IgM capture, and this ensures that no cross reactivity occurs with IgG, IgA, or IgE (Duffy *et al*., 1989). However, the persistence of IgM antibodies specific to *T. gondii* may compromise interpretation of results by such assays.

2.7.1.5. Enzyme-Linked Immunosorbent Assay (ELISA)

The detection of anti-*T. gondii* specific antibody is considered the first line in diagnosis of acute toxoplasmosis (Beghetto *et al*., 2006). Different serological based methods for detecting *T. gondii* by ELISA have been developed and are widely used. However, these methods have limitations such as failure to detect the IgG and IgM anti-*T. gondii* specific antibodies early enough upon development of parasitemia (El Gamal *et al*., 2013). Previous studies have reported false positive and false negative results in IgG and IgM ELISA tests when compared with PCR. Furthermore, some ELISA tests have shown the presence of IgG and IgM *T. gondii* antibodies in

a single serum sample and therefore interpretation of such results may be difficult (El Gamal *et al*., 2013; Howe and Sibley, 1995).

2.7.2. Histological techniques

In humans, histological techniques have been used to demonstrate tachyzoites in tissue sections and smears prepared from body fluids such as amniotic fluid and cerebrospinal fluid (Remington *et al*., 2001). *T. gondii* has also been demonstrated in Giemsa stained preparations of sediments from brain aspirates, cerebrospinal fluid and biopsy tissue impression smears (Montoya, 2002). However, this is not a widely used technique.

2.7.3. Other alternative diagnostic methods

T. gondii can be demonstrated by imaging techniques such as computerized tomography (CT) for identification of small lesions located in the posterior fossa (Markus, 2003; Sukthana, 2006 Vidal *et al*., 2005). However the method is costly and can only be performed by skilled physicians**.**

2.7.4. Molecular methods of detection

Most amplification methods such as ligase chain reaction (LCR), nucleic acid sequence-based amplification (NASBA), and strand displacement amplification (SDA) have the potential for use as kits for detection of *T. gondii*. The methods have been introduced for routine diagnostics as nucleic acid amplification tests (NATs) for DNA detection (Versalovic & Lupski 2002). For instance LCR and NASBA have been used for detection of Plasmodium (McNamara *et al*., 2004; Schneider *et al*., 2005). The methods have additional advantages over conventional methods since they have a mechanism that facilitates standardization and automation coupled with ability to type species and detect drug resistance (Dong, 2008).

Alternative molecular techniques such as Polymerase Chain Reaction (PCR) and the Loop Mediated Isothermal Amplification (LAMP) of DNA have also been developed and evaluated using human and animal samples (Lau *et al*., 2010; Kong *et al*., 2012; Zhang *et al*., 2009). Other research based next generation diagnostic methods using DNA sequencing, bioinformatics and phylogenetic analysis hold great promise for genetic identification of *T. gondii*.

2.7.4.1. Polymerase Chain Reaction (PCR)

The use of PCR in *T. gondii* DNA amplification has revolutionized diagnosis of toxoplasmosis when compared to serological detection techniques (Alfonso *et al*., 2009). Different types of PCR reaction such as the standard PCR, nested PCR and Real time PCR have been used to amplify various *T. gondii* DNA targets. Previous studies have shown that PCR with primers based on multi-copy elements such as B1 and 529bp repeats give better results when compared with single gene targets such as SAG1and SAG 2 (Vidal *et al*., 2004; Colombo *et al*., 2005). Nested PCR and Real-time PCR have been reported as more superior for the detection of *T. gondii* DNA compared to standard PCR. For instance, the increased sensitivity and specificity in nested PCR is due to the re-amplification of a small section of primary PCR product of amplification by use of a different pair of PCR primers. Real-time PCR has been used to amplify and quantify the DNA which can aid in the enumeration of parasites in different tissues and fluid samples obtained from humans and animals (Al-nasrawi *et al*., 2014). However, PCR methods are not used widely due to high cost of apparatus and long reaction time (Gross *et al*., 1992).

The recently described 529bp DNA non-coding fragment of *T. gondii* occurs in much higher copies in the range of 200-300 compared to the 35-fold B1 gene (Kong *et al*., 2012). The 529bp multicopy DNA is also a highly conserved at nucleotide sequence between the strains as well as the isolates. The DNA target provides a great improvement in the PCR test sensitivity for *T.*

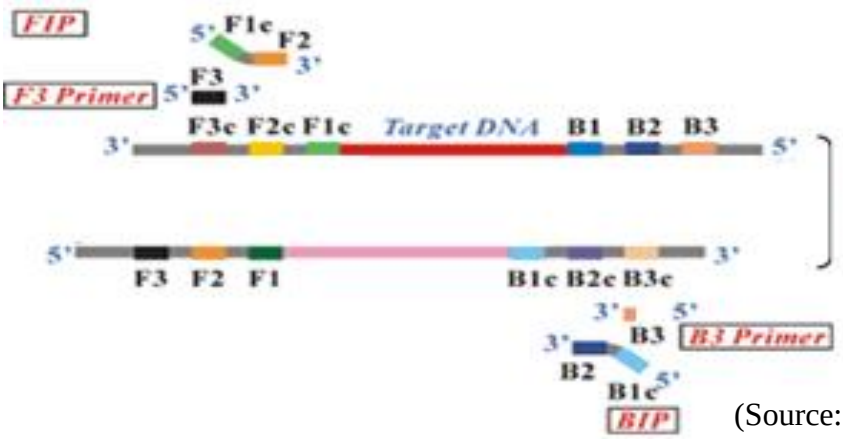
gondii DNA detection, compared to B1gene and single copy targets **(**Homan *et al*., 2000; Edvinsson *et al*., 2006). The B1gene has been widely used in epidemiological studies and the sensitivity has been reported to be similar to that of ITS-1 and 18S rDNA.

2.8. Loop Mediated Isothermal Amplification (LAMP)

LAMP is a nucleic acid amplification technique that synthesizes large amounts of DNA in short period of time and has high specificity (Mori *et al*., 2001).

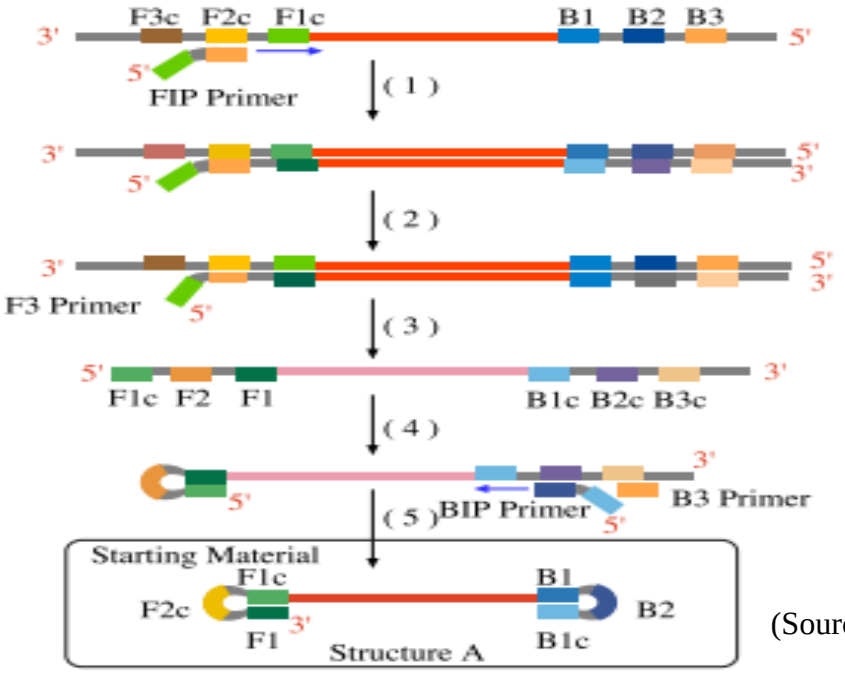
2.8.1. The basic mechanism of the LAMP method

The LAMP is a one-step amplification reaction of a target DNA sequence under isothermal conditions usually between 60 °C and 65 °C. Basically, the LAMP reaction involves three steps, an initial step, a cycling amplification step, and an elongation step (Notomi *et al*., 2000; Mori & Notomi, 2009). LAMP usually employs Bst DNA polymerase that has a strand-displacement activity, two inner primers (FIP, BIP) and two outer primers (F3, B3) that recognize six separate regions (F3c, F2c, F1c, B3c, B2c and B1c) in a target DNA as shown in Fig. 2.4. The initial step in LAMP reaction yields the starting structure as shown in Fig. 2.5. The loop primers (LF and LB) shown in Fig. 2.6, may be included and recognize another two separate regions of the elongated structure to accelerate the LAMP reaction (Nagamine *et al*., 2002).



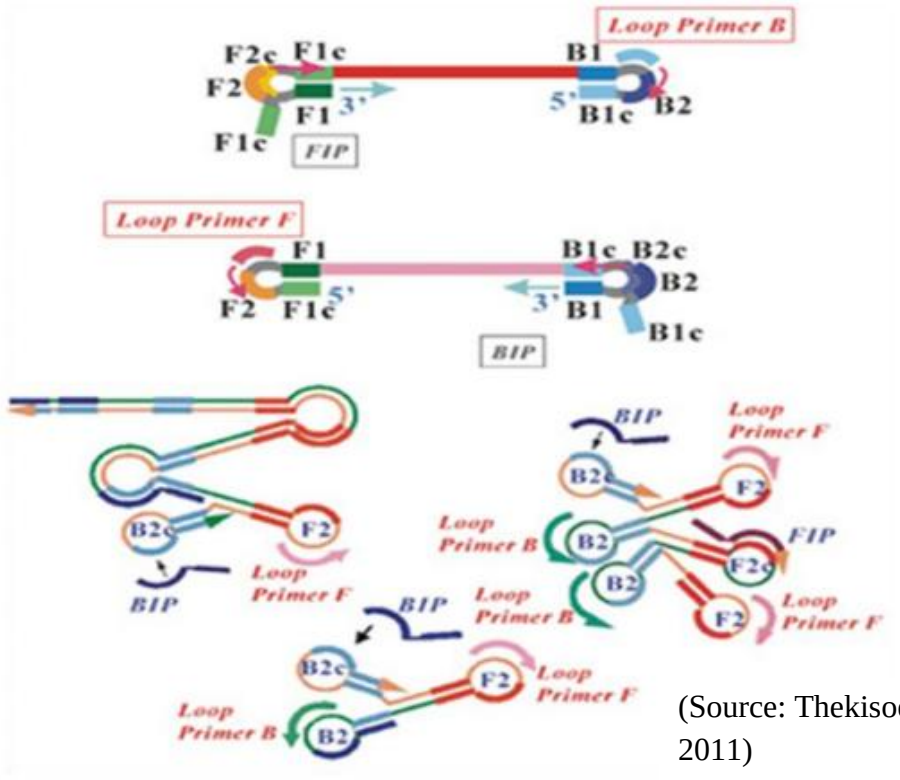
(Source: Thekisoe & Inoue, 2011)

Fig. 2.4. Chart showing inner and outer primer binding sites on target fragment



(Source: Mori *et al*., 2004)

Fig. 2.5. Chart showing initial step of LAMP reaction



(Source: Thekisoe & Inoue, 2011)

Fig. 2.6. Chart showing binding sites of loop primers in cycling and elongation steps of LAMP reaction

2.8.2. Advantages of LAMP technique over serology and PCR

During primary infection, seroconversion may not be differentiated from sero-reversion by serological methods following latent infection (van Druten *et al*., 1990). On the other hand, immunosuppressive conditions may affect the kinetics of IgG avidity maturation (Lutz *et al*., 1994). Pregnancy, possibly augmented by antitoxoplasma pharmacotherapy, may lead to a similar effect (Sensini, 2006). Such limitations can be overcome by LAMP technology which has a great potential over serologic and most DNA-based amplification assays.

LAMP is a very sensitive, easy and less time consuming method. The LAMP method can amplify between 10^9 and 10^{10} copies of DNA template (Notomi *et al*., 2000), in less than an hour

under isothermal conditions (65 ºC). A simple incubator, such as a water bath or block heater, is sufficient for DNA amplification, hence feasibility for application in the field conditions (Boehme *et al*., 2007).

The LAMP test demonstrates high tolerance to biological products (Kaneko *et al*., 2007), implying that DNA extraction may not be necessary (Poon *et al*., 2006), and the product can be observed visually on addition of SYBR Green I (Iwamoto *et al*., 2003, Yoshida *et al*., 2005). The health risks posed by the use ethidium bromide in agarose gel electrophoresis in PCR may be avoided if LAMP is adopted.

LAMP reactions can be run with reagents stored at 25^{o}C and 37^{o}C, which make it applicable in the tropical and sub-tropical countries where protozoan diseases including *T. gondii* are common (Thekisoe *et al*., 2009). LAMP has been shown to successfully amplify trypanosome DNA with reagents stored at the above stated temperatures (Thekisoe *et al*., 2009). Bst DNA polymerase and the LAMP buffer solution are relatively stable when not in the cold chain (Njiru, 2012). It has been confirmed that LAMP has superior tolerance to inhibitors when compared to PCR, since PCR was inhibited by minimal concentrations of hemoglobin, IgG and IgM, while none inhibited LAMP (Thekisoe *et al*., 2009). The stability of LAMP reagents adds to the advantages of the method and raises possibility for being implemented in poor resourced diagnostic laboratories.

2.8.3. Detection of LAMP amplification product

The large amount of LAMP amplification product normally generated in a LAMP reaction can be detected by use of methods such as the real-time turbidimeters and fluorescent dyes (Notomi *et al*., 2000). In LAMP reactions, the use of mutagenic dyes such as ethidium bromide can be

avoided as the end results can be judged in a closed tube. This has also been reported to reduce contamination of the working environment which is commonly associated with manipulation of DNA during electrophoresis.

2.8.3.1. Real-time detection

Real-time turbidity measurement by use of photometer with incubation function has enabled the kinetic analysis of the LAMP reaction without the need for any detection reagents such as a fluorescence intercalator. The LAMP reaction has the ability to produce insoluble magnesium pyrophosphate in the course of the amplification reaction. The real-time turbidity analysis of LAMP makes it possible to quantify the initial amount of template DNA in the samples (Mori, 2004).

2.8.3.2. Visual inspection

LAMP positive reaction can be easily detected by visual endpoint judgment of turbidity (Mori, 2001), on addition of SYBR Green I (Iwamoto *et al*., 2003; Yoshida *et al*., 2005). Recently, an effective endpoint detection method has been developed by using calcein, whose fluorescence is quenched by the binding of manganese ions (Tomita *et al*., 2008). Because the manganese ions are bound by pyrophosphate ions produced in the course of the amplification reaction, calcein produces bright fluorescence in a positive reaction (Tomita *et al*., 2008).

2.8.4. LAMP assay for the detection of *T. gondii*

The feasibility of LAMP technique in clinical diagnosis of toxoplasmosis is currently being studied and evaluated at research level (Kong *et al*., 2012). The developed SAG1-based LAMP has exhibited a degree of sensitivity greater than the ordinary PCR, qualifying LAMP method as

a more convenient and sensitive diagnostic kit for routine health control of *T. gondii* infection (Krasteva *et al*., 2009).

Previous studies have demonstrated the presence of *T. gondii* in infected mouse organs by *SAG1*-LAMP, and this may indicate reliability of the method to detect *T. gondii* in biopsy specimens (Krasteva *et al*., 2009). LAMP assays targeting the *B1*, *SAG1*, and *SAG2* genes have been developed for detection of *T. gondii* DNA (Lau *et al*., 2010). Another target known as the 529bp repetitive element has been reliably used for detection of *T. gondii* DNA extracted from lymph nodes and blood samples obtained from animals (Zhang *et al*., 2009; Kong *et al*., 2012).

2.8.4.1. LAMP assay targeting 529bp fragment

The LAMP assay based on the conserved 200 to 300-fold 529bp repetitive element has shown a higher sensitivity than conventional PCR (Zhang *et al*., 2009). The method was used to detect *T. gondii* in blood samples of laboratory infected mice (Kong *et al*., 2012). As a result of the high sensitivity, specificity and rapidity of LAMP, the method has been suggested as one of the most accurate molecular assays for the early detection of *Toxoplasma* in blood samples. Application of the 529bp LAMP assay on clinical samples such as serum has not yet been conducted and thus will be determined in this study.

2.8.4.2. Improvement of LAMP

The recommendations by the World Health Organization (WHO) are that a reliable diagnostic test suiting developing world should be affordable in terms of cost. The test should also be sensitive, specific, easy to use, rapid and robust, requires no equipment, and is deliverable (Mabey *et al*., 2004). LAMP technology is the most suitable method among the limited methods

of DNA amplification. However, LAMP application under field conditions is limited, initially owing to the infancy of the technologies linked to it, including field-based methods of template preparation and modes of product detection. In order for the LAMP assay to demonstrate its performance objectives, some technologies tied to LAMP test need to be developed. These include a lyophilized reagent mix, DNA template preparation protocols that are short such as boiling of sample, a reliable power source such as use of car battery, and product detection techniques by use of fluorescent dyes (Njiru, 2012).The use of fluorescence dyes such as SYBR green I in the detection of *T. gondii* DNA by LAMP technology has been reliably performed (Kong *et al*., 2012). However, the other protocols that need to be developed in LAMP have not been fully developed. In this study, the performance of lyophilized reagent for the detection of *T. gondii* DNA was evaluated as a step towards transformation of LAMP for field application.

2.8.4.3. Lyophilized reagent mix

A standard LAMP reaction consists of a large amount of reaction components, ranging from the enzyme, its buffer, deoxynucleotide triphosphates, betaine, four to six primers of different concentrations and the detection dye. In addition to the heavy task of acquiring different reagents from varied sources usually faced by the researcher or technician, the requirement for the cold chain, a major cost during transportation and storage still exists (Njiru, 2012). Studies conducted previously indicate that LAMP reagents can remain stable at temperatures of up to 37 °C, indicating the prospects for lyophilization (Thekisoe *et al*., 2009). Recently, lyophilized kits such as those used for detection of *T. b. brucei* and *Plasmodium spp*. have been launched and are under evaluation (Mitashi *et al*., 2013; Hopkins *et al*., 2013). The use of lyophilized LAMP kits could further simplify the LAMP technique and the reactions can be performed without the need for prior preparation of reagents of LAMP in the laboratory.

CHAPTER 3

3.0 MATERIAL AND METHODS

3.1. Study group

The study group composed of licensed abattoir workers in Thika District. The baboons (*P. anubis*) previously trapped in Thika district were also included in the study. Thika District covers 1,960.2 sq Km² (lies between 3°53′and 1°45′S and 36°35′and 37°25′East) and the study sites were as shown Fig.3.1.

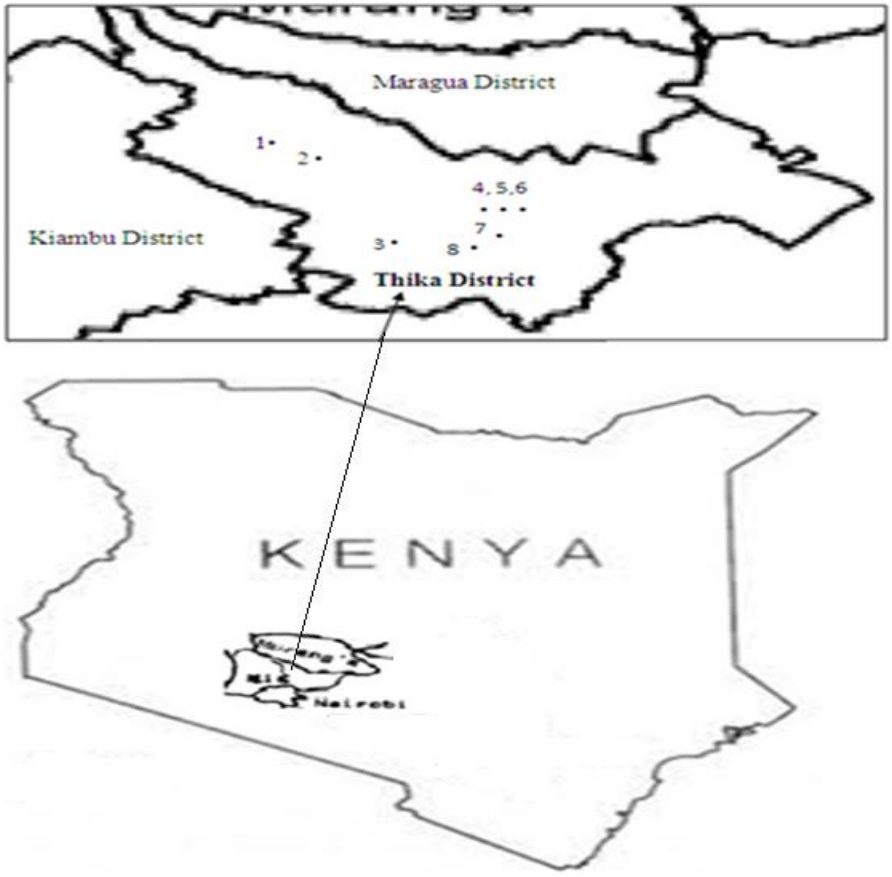


Fig.3.1. Map of Thika District showing its location in the map of Kenya and the 8 study sites

In Fig 3.1, the study site are represented by numbers 1 to 8 as follows: 1. Mundoro slaughter slab, 2. Gachika slaughter slab, 3. Gathage slaughter slab, 4. Thika slaughter slab, 5. Thika Municipal slaughter house,6. Thika chicken slaughter house, 7. Juja slaughter slab and 8. Ruiru slaughter house.

3.2. Study design

This was a laboratory based study using stored human and baboon (*P. anubis*) serum samples.

3.3. Ethical approval

The permission to undertake the study in human beings was obtained from the Institutional Ethical Review Committee of Kenya Medical Research Institute (KEMRI) (Appendix 1). The workers were informed about the study objectives and those who gave written consent were included in the study. Moreover, the permission to undertake the study in baboons (*P. anubis*) was obtained from the Institutional Ethical Review Committee of Institute of Primate Research (IPR) (Appendix 2).

3.4. Sample collection

3.4.1. Human

Using the standard venipunture technique, 4ml of human blood samples were collected from a total of 87 consenting workers between March and June 2013.

3.4.2. Baboons

A total of 87 baboons (*P. anubis*) (38 males and 49 females), comprising 26 adults and 61 juveniles captured over time from Kimandi, Kakuzi and Yatta areas near Thika District between 2006 and 2011 were studied. The animals were kept under quarantine at the Institute of Primate Research, a directorate of the National Museums of Kenya (NMK) located in Karen near

Nairobi. While still under quarantine, the animals were intravenously sedated with xylene-ketamine solution (Interchemie Bv, Holland and Kepro Bv, Holland) and 5 ml of blood was collected by femoral venipuncture.

3.5. Sample processing

The blood samples were transferred to labeled sterile tubes and immediately stored in upright position in refrigeration cool box. The blood samples were transported to the laboratory within 5 hours of collection, stored upright at +4 ^{o}C overnight before separation of serum. Serum was separated from the blood samples by pipetting and samples stored in aliquots of 200µl at -20 ^{o}C until analyzed as indicated below.

3.6. DNA isolation

DNA was extracted from the serum samples using Quick-gDNA^{TM}MiniPrep kit (Zymo research, U.S.A) according to the manufacturer's instructions. At room temperature, 100µl of serum sample was added to 400µl of genomic lysis buffer, briefly vortexed and allowed to stand for 5 min. The mixture was transferred to a zymo-spin column in a collection tube and centrifuged at 10000 rounds per min (RPM) for 1 min. The zymo-spin was transferred to a fresh collection tube and a DNA pre-wash buffer (200µl) added, followed by centrifugation at 10000 rounds per min (RPM) for 1 min. To the same spin column, 500µl of g-DNA wash buffer was added, followed by centrifugation (10000 RPM) for 1 min. The spin column was transferred to a clean microcentrifuge tube and 60µl of elution buffer added. The eluted DNA was stored at -20 ^{o}C in DNase-free eppendorf tubes.

3.7. Positive control

Reference *T. gondii* (RH) DNA was kindly donated by the Friedrich-Loeffler-Institut Bundesforschungs institute für Tiergesundheit based in Germany through the assistance of Dr. Gereon Schares based in the same institute.

3.8. PCR technique

Nested PCR targeting the 529bp multi-copy gene element (GenBank Accession no.AF146527) was performed as previously described (Kong *et al*., 2012). The reaction mixture for primary amplification was optimized in 10µl reaction volume. The components of the PCR reaction mix included 2µl of 10x Dream Taq buffer (Thermo Scientific, Waltham, MA USA), 0.15µl of 5µM of each of the primer pair 5'TGACTCGGGCCCAGCTGCGT3'and 5'CTCCTCCCTTCGTCCAAGCCTCC3', 0.5µl of Dream Taq polymerase (Thermo Scientific, Waltham, MA USA), 0.5µl of DNA template, 0.2µl of 10mM dNTP mix and then topping the reaction volume with double distilled water. The PCR reaction was run for 30 cycles of denaturation temperature at 94 ^{o}C for 1 min, annealing at 58 ^{o}C followed by an extension at 72 ^{o}C.

In the secondary amplification, the primary PCR amplification product was diluted 1:99 using double distilled water. From the dilution product, 0.5µl was used as template. The reaction mixture composed of 2.1µl of 10x Dream Taq buffer (Thermo Scientific, Waltham, MA USA), 0.5µl of 5µM of each primer pair 5'AGGGACAGAAGTCGAAGGGG3' and 5'GCAGCCAAGCCGGAAACATC3'. A volume of 1µl of Dream Taq polymerase (Thermo Scientific, Waltham, MA USA) was added and the reaction volume topped up to 10µl with molecular grade water for PCR assay.

PCR reaction was then run for 35 cycles of denaturation temperature at 94 ^{o}C for 1 min, annealing at 52 ^{o}C followed by an extension at 72 ^{o}C. The reference *T. gondii* (RH) DNA was used as positive control, while PCR water was used as the negative control. The two controls were included in every amplification step. The product generated in the second amplification was run in 1.5% agarose gel pre-stained with 3µl of ethidium bromide (1µg/ml).

3.9. Loop Mediated Isothermal Amplification technique

A total of six primers targeting eight regions of the 529 bp repetitive element were used in the LAMP reaction. The six LAMP primers were outer forward (F3), outer backward (B3), forward inner primer (FIP), backward inner primer (BIP), loop forward (LF) and loop backward (LB). The sequences for the six LAMP primers in Table 3.1 are as described previously by Zhang *et al* (2009).

Table3.1. LAMP primer sequences

LAMP Primer	Sequence
F3	5'CCACAGAAGGGACAGAAGTC3'
B3	5'TCCGGTGTCTCTTTTTCCAC3'
FIP	5'TCCTCACCCTCGCCTTCATCTAGGACTACAGACGCGATGC3'
BIP	5'TGGTTGGGAAGCGACGAGAGTTCCAGGAAAAGCAGCCAAG3'
LF	5'TCCAAGACGGCTGGAGGAG3'
LB	5'CGGAGAGGGAGAAGATGTTTCC3'

(Source: Zhang *et al*., 2009)

3.8.1. The wet LAMP

The LAMP assay targeting the 529 bp multi-copy gene element (GenBank Accession no. AF146527) was performed as described by Zhang *et al*., (2009), but with a few modifications such as adjustment of volumes of reaction mixture components. The master mix for the LAMP

reaction was prepared and aliquots of 24 µl were made to contain the reaction components which included 1µl of Bst DNA polymerase, 1µl of template DNA, 40pmol each of FIP and BIP primer, 5pmol each of F3 andB3, 20pmol each of LF and LB, 1x LAMP reaction buffer (20 mM Tris–HCl (pH 8.8), 50mM KCl, 10 mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, and 0.1% Tween-20) and 1ul of 10mM dNTP mix. The reaction volume was topped up to 25 µl with PCR water (Zhang *et al*., 2009). Incubation was done in loopamp real-time turbidimeter (LA-320CE, 4- 19, TAITO, TAITO-KU, TOKYO 110-8408, Japan). The LAMP reaction was inactivated by incubation at 80^oC for 2 min. The reference *T. gondii* (RH) DNA was used as positive control, while PCR water was used as the negative control in every amplification step. The specificity of the LAMP test was determined by use of non-*T. gondii* DNA from other Apicomplexan parasite (*Trypanosoma brucei brucei* and *Theileria parva*), obtained from the Institute of Primate Research.

3.8.2. Detection of LAMP amplification product

For the wet LAMP, the LAMP product was progressively generated and qualitatively measured by real time turbidimeter. After 1hr of LAMP reaction, electrophoresis was run in 1.5% agarose gel pre-stained with 3µl of ethidium bromide (1µg/ml) as a confirmatory test.

3.8.3. Lyophilization of the LAMP master mix

The master mix was prepared as described in section 3.8.1. Aliquots of 24µl of the master mix were dispensed in DNase-free 0.2ml PCR tubes. The PCR tubes were capped and two holes made in each cap by use of DNase free 22-gauge needle. The PCR tubes were placed in upright position in DNase-free plastic rack, which was later sealed. The tubes were transferred to the deep freezer and the master mix was frozen at -80^o C for 5min. The frozen master mix was then transferred to the freeze drying chamber of freeze dryer (Telstar, 37583: LyoAlfa Plus 10-55,

Terrassa, Spain). Freeze dying of the master mix was performed at 0.400mBar of vacuum pressure, at -50^{o}C for 1hr. The freeze dried master mix was removed from the drying chamber and capped with DNase-free caps. The freeze dried reagent was stored overnight at room temperature.

3.8.3.1. The dry LAMP

To the reaction tube, 24 µl of template DNA was added to reconstitute the dry LAMP reagent. The reaction mixture was briefly vortexed followed by incubation as described in section 3.8.1. The negative control (PCR water) and positive control (*T. gondii* (RH) DNA) were also included.

3.9. Statistical analysis

The Fisher's exact test and was used to determine any significant difference in detections between nested PCR and wet LAMP, between lyophilized LAMP and nested PCR and also between lyophilized LAMP and wet LAMP. The same test was used to determine the difference in *T. gondii* positivity between the chicken slaughter house workers and the ruminant (cow-sheep-goat) slaughter house workers. The difference in *T. gondii* positivity between the two age categories of baboons was also determined by the same test. A value was considered significant at $P<0.05$ with a 95% confidence interval using the Epi-info software version 6 (CDC, Atlanta).

CHAPTER 4

4.0 RESULTS

4.1. Detection of *T. gondii* DNA in human serum by nested PCR

The primary PCR product was characterized by multiple bands of various sizes, some with sharp and faint bands equivalent to 400 bp being observed in some samples and the positive control as shown in fig. 4.1. Secondary amplification product clearly showed the predicted amplicon size of 164 bp as shown in Fig. 4.2.

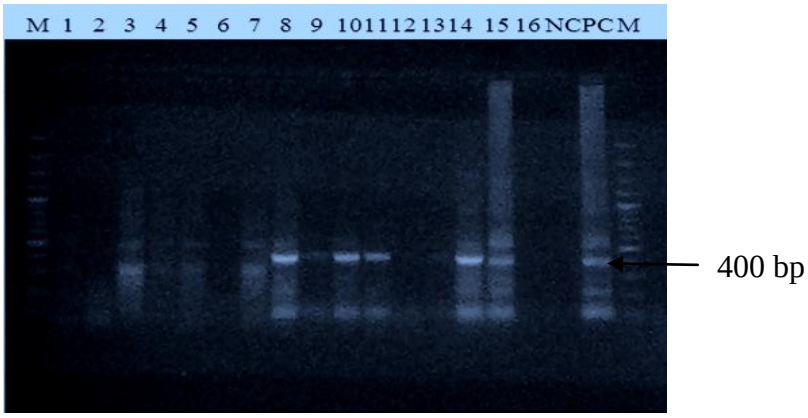


Fig.4.1. Gel photograph for primary PCR amplification product in human serum

In Fig.4.1, the human serum samples are shown by lanes 1-16 as representative of the 87 human samples. NC and PC denote negative (PCR water) and positive (*T. gondii* RH DNA) controls respectively. Lane M denotes the 100bp Molecular ruler.

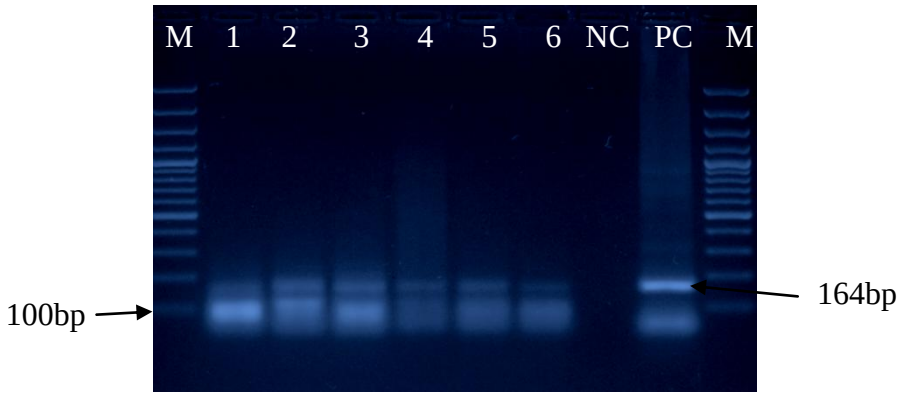


Fig.4.2. Gel photograph for secondary PCR amplification product in human serum

In Fig.4.2, the human serum samples are represented by lanes 1-6. NC, PC & M denotes negative (PCR water) and positive (*T. gondii* RH DNA) controls and 100bp ladder respectively.

In overall, 39.1% (34/87) of the slaughter house and slaughter slab workers tested positive for T. gondii. The parasite was detected in workers from most (6/8) of the slaughter houses except from Mundoro and Gachika slaughter slabs as shown in Table 4.1. Workers in chicken slaughter house in Thika recorded the highest prevalence (100%) followed by Thika slaughter slab with 60%. The proportion of *T. gondii* in workers at the chicken slaughter house was significantly higher than those workers in ruminant (cattle-sheep-goat) slaughter house (p=0.003).

Table 4.1. Number and percentage of male workers who tested positive for *T. gondii* DNA using nested PCR

Study site	Type of livestock slaughtered	Workers tested positive (%)
Thika chicken slaughter house	Chicken	6/6 (100)
Thika slaughter slab	Cattle, sheep, goat	6/10 (60)
Ruiru slaughter house	Cattle, sheep, goat	10/22 (45.5)
Juja slaughter slab	Cattle-sheep-goat	6/15 (40)
Thika Municipal slaughter house	Cattle-sheep-goat	5/14 (35.7)
Gathage slaughter slab	Cattle-sheep-goat	1/5 (20)
Mundoro slaughter slab	Cattle-sheep-goat	0/10 (0)
Gachika slaughter slab	Cattle-sheep-goat	0/5 (0)
Overall		34/87 (39.1)

4.2. Detection of *T. gondii* DNA in baboon serum samples by nested PCR

The analysis of baboon serum samples showed that 33.3% (29/87) of the baboons tested were infected with *T. gondii*. The agarose gel electrophoresis of the nested PCR product showed bands equivalent to 164 bp as shown in Fig. 4.3. The bands were only present in positive control and the positive samples.

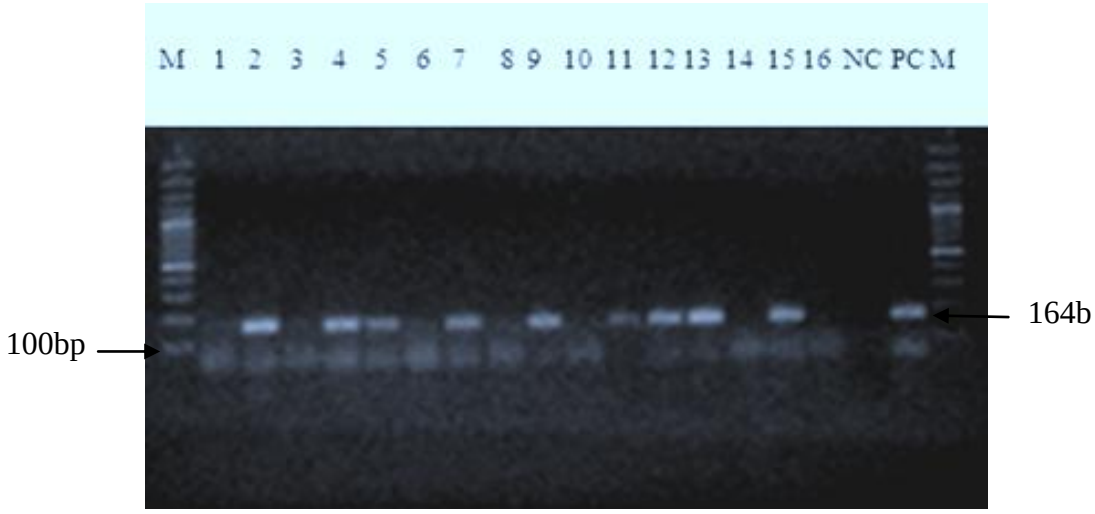


Fig.4.3. Gel photograph for secondary PCR amplification product in baboon serum

In Fig.4.3, Lanes 1 to16 represent baboon samples (representative) while lanes NC, PC and M denote negative control (PCR water), positive control (*T. gondii* RH DNA) and 100bp molecular ladder, respectively.

The prevalence of *T. gondii* was higher (58.6%) in adult compared to the juvenile baboons as shown in Table 4.2. However, there was no significant difference (p= 0.63) in *T. gondii* infection between the juvenile and the adult baboons.

Table 4.2. Proportions of juvenile and adult baboons infected with *T. gondii* as tested by nested PCR

Age	**Baboons tested positive (%) by nested PCR**
Juvenile (1-5 years)	41.38 (12/29)
Adult(>6 years)	58.62 (17/29)

4.3. Performance of wet LAMP reagents in real time detection of *T. gondii* DNA

The LAMP amplification produced detectable amounts of LAMP amplification product as seen by increase in turbidity shown in Fig.4.4 and Fig. 4.5. The detection time of LAMP amplification product was between 20 and 60min. The negative control, DNA of other parasites (*T. b. brucei* and *T. parva*), were not amplified.

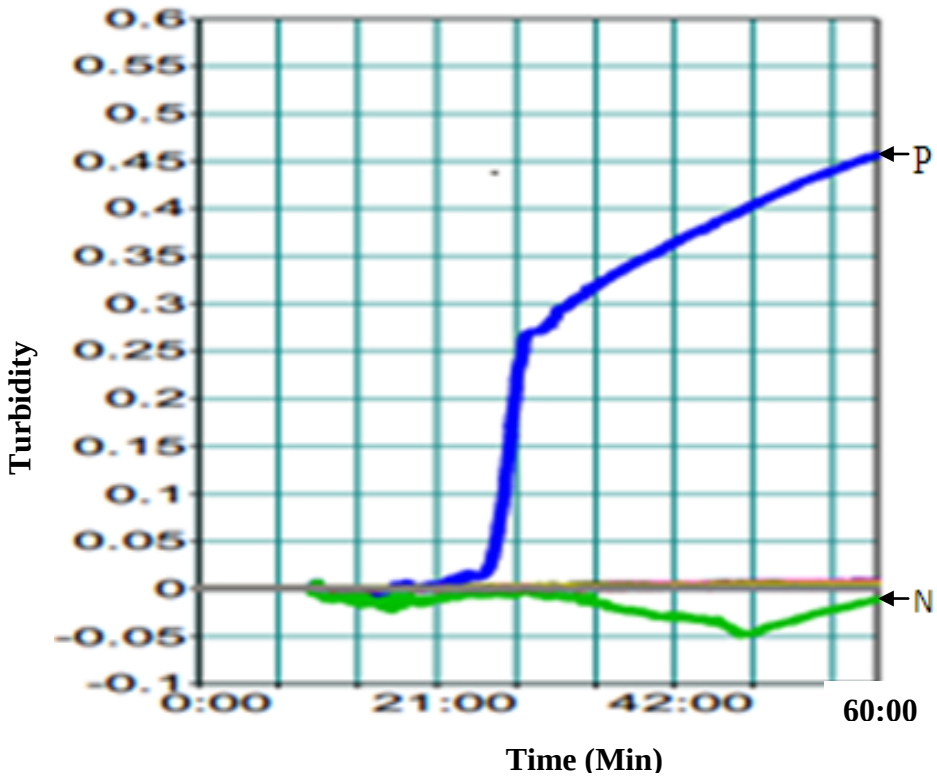


Fig. 4.4. Graph for real-time LAMP amplification using turbidimeter

In Fig. 4.4, P and N denote the positive (*T. gondii* RH DNA) and negative controls (PCR water), respectively.

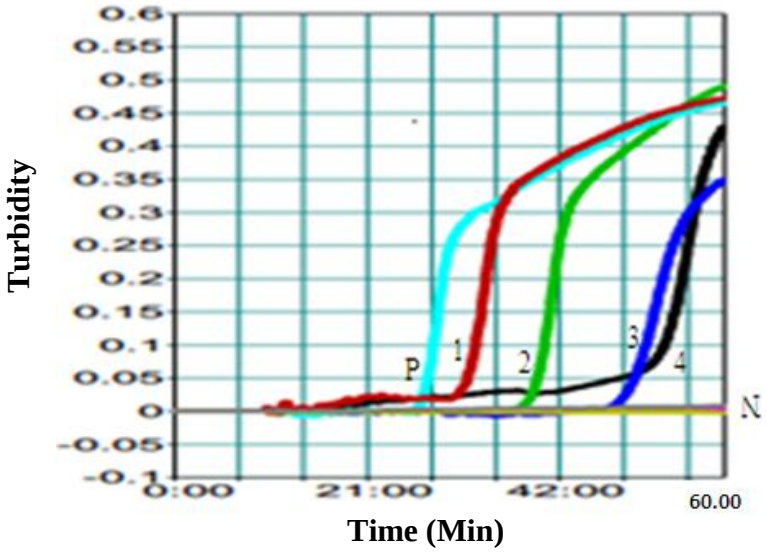


Fig. 4.5. Turbidimetric LAMP amplification curves showing representative human serum samples

In Fig. 4.5, lanes 1-4 show the human serum samples, while P and N denote the positive (*T. gondii* RH DNA) and negative (PCR water) controls.

The agarose gel electrophoresis of the amplification product generated by the LAMP reaction showed characteristic ladder-like bands in positive control and samples as shown in Fig. 4.6. No LAMP product was detected in the negative control.

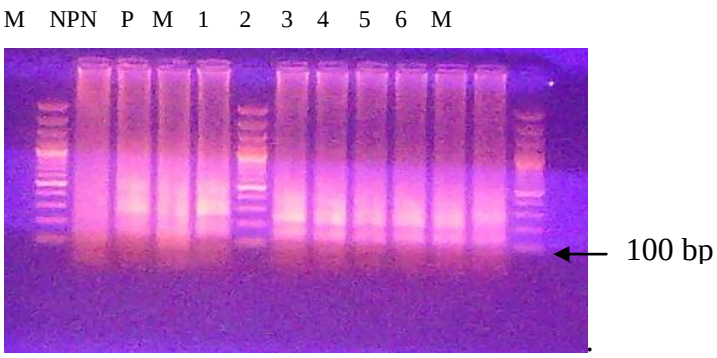


Fig. 4.5. Gel photograph for LAMP amplification product in human serum

In Fig. 4.6, lanes 1-6 are human serum samples (representative). Lanes N, P and M denote negative control (PCR water), positive control (*T. gondii* RH DNA) and100bp molecular ladder, respectively.

In overall, 33.3% (29/87) of the slaughter house workers tested positive for *T. gondii* DNA by wet LAMP. As shown in Table 4.3, the chicken slaughter house workers showed the highest infection rates (83.3%) followed by Ruiru slaughter house workers (50%). In Thika, 40% of the slaughter house workers were infected with *T. gondii*.

Table 4.3. *T. gondii* positivity in slaughter house workers by wet LAMP and dry LAMP

Study site	Type of livestock slaughtered	Workers tested positive (%) by wet LAMP	Workers tested positive (%) by dry LAMP
Thika chicken slaughter house	Chicken	5/6 (83.3)	6/6 (100)
Ruiru slaughter house	Cattle, sheep, goat	11/22 (50)	13/22 (59.1)
Thika slaughter slab	Cattle, sheep, goat	4/10 (40)	5/10 (50)
Juja slaughter slab	Cattle-sheep-goat	4/15 (26.7)	5/15(33.3)
Thika Municipal slaughter house	Cattle-sheep-goat	3/14 (21.4)	6/14 (42.9)
Gathage slaughter slab	Cattle-sheep-goat	1/5 (20)	0/5 (0)
Gachika slaughter slab	Cattle-sheep-goat	1/5 (20)	1/5 (20)
Mundoro slaughter slab	Cattle-sheep-goat	0/10 (0)	1/10 (10)

4.4. Performance of dry LAMP in the detection of *T. gondii* DNA

The lyophilization process of the master mix failed to yield a completely dried master mix for dry LAMP due to glycerol present in Bst polymerase formulation used. However, when detection of *T. gondii* DNA by dry LAMP reagent was applied, 42.5% (37/87) of the slaughter house workers were shown to be infected. The chicken slaughter house workers in Thika showed the highest proportion of the workers (100%) with detectable parasite DNA followed by Ruiru slaughter house workers (59.1%) as shown in Table 4.3. Twenty percent of the samples collected from Gachika slaughter slab workers had detectable parasite DNA while 10% of those samples

obtained from Mundoro slaughter slab workers had the parasite DNA. However, the dry LAMP failed to detect *T. gondii* DNA in samples obtained from Gathage slaughter slab workers.

4.5. Comparison of the performance of the three tests in *T. gondii* DNA detection

In overall, nested PCR detected *T. gondii* DNA in 39.1% of the slaughter house workers while, dry LAMP showed that 42.5% of the workers were infected with *T. gondii*. In Ruiru alone, nested PCR detected *T. gondii* DNA in 45.5% of the slaughter house workers while dry LAMP showed that 59.1% of the workers were infected with *T. gondii*. The sensitivity of nested PCR was higher than dry LAMP in two study sites as shown in Fig. 4.6. The dry LAMP detected *T. gondii* DNA in Gachika and Mundoro which was not detected by nested PCR. However, there was no significant difference (p=0.83) in performance between nested PCR and dry LAMP.

4.6. Comparison of the performance of dry LAMP with wet LAMP in *T. gondii* DNA detection

The dry LAMP showed higher sensitivity to *T. gondii* DNA than wet LAMP in almost all the study sites (6/8). The wet LAMP showed lower sensitivity (83.3%) to *T. gondii* DNA in the chicken slaughter house workers compared to dry LAMP (100%). However, there was no significant difference ($p = 0.92$) in performance between the wet and dry LAMP.

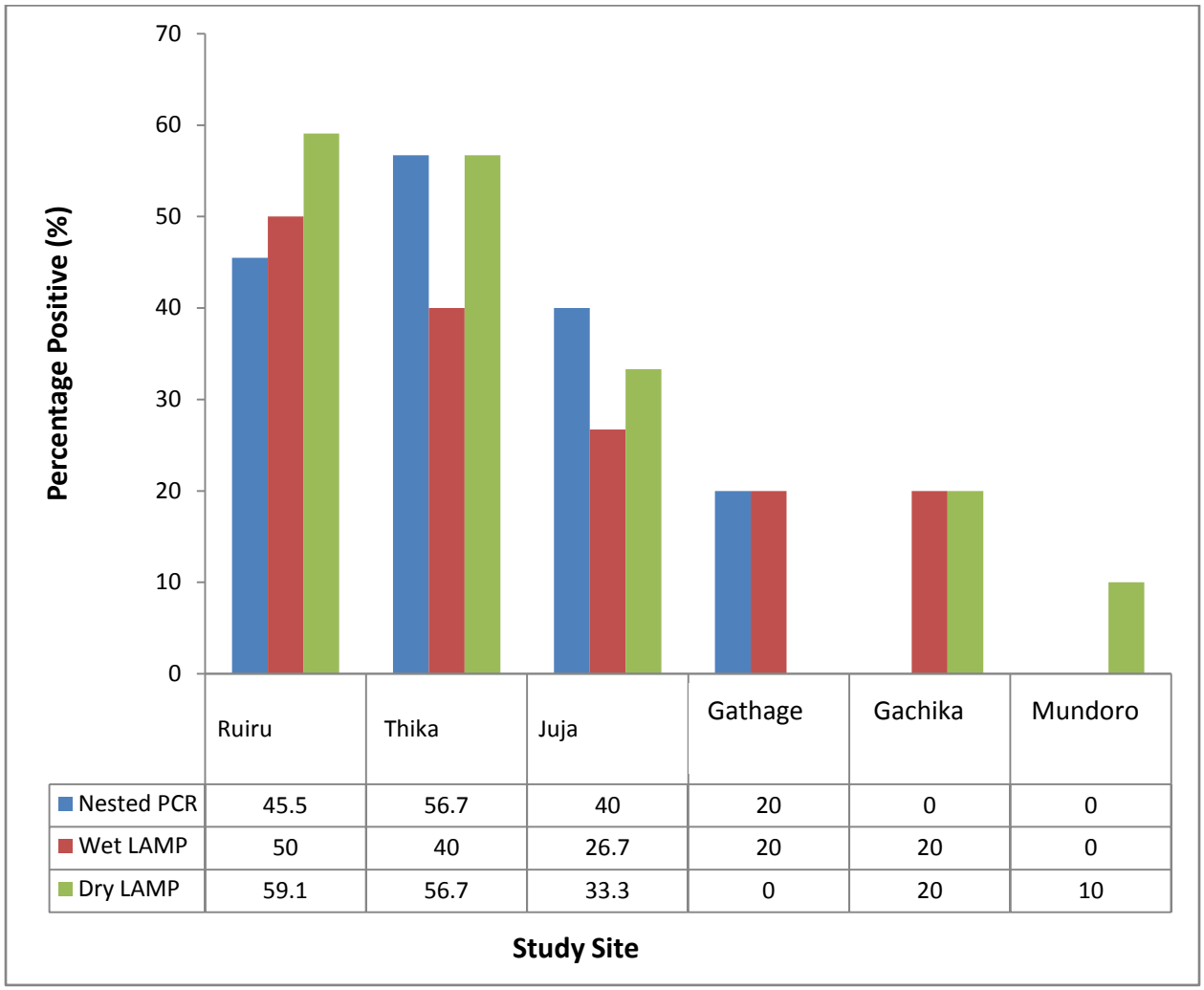


Fig. 4.6. The detection of *T. gondii* in different study sites by various DNA amplification methods

In Fig. 4.6, the graph shows a summary of the proportions of *T. gondii* infected slaughter house workers in 6 study area as tested by nPCR, wet LAMP and dry LAMP. The slaughter house workers in Thika were combined to form one study site.

CHAPTER 5

5.0 DISCUSSION

The current study used molecular based assays to determine the prevalence of *T. gondii* in human and baboons from Thika District. In this study, nested PCR targeting the 529bp repeat element was found to amplify *T. gondii* DNA from human and baboon serum samples. Previous studies have reported that nested PCR is highly specific for *T. gondii* DNA using samples such as cerebrospinal fluid (CSF), amniotic fluid, fetal and neonatal blood, suggesting that this technique could be used for the detection of *T. gondii* (Tlamçani *et al*., 2013; Menotti *et al*., 2010; Wallon *et al*., 2010).

The current study demonstrated that 39.1% of the slaughter house workers were infected with *T. gondii* as indicated by the detection of the parasite DNA in human serum samples. Higher sero-prevalences of *T. gondii* have been reported in pre-school children (35-60%) and human blood donors (54%) in Nairobi County at Kenyatta National Hospital (Bowry *et al*., 1986; Griffin & Williams, 1983). The present study used serum samples obtained from asymptomatic abattoir workers. Reduction or absence of *T. gondii* parasites in peripheral blood has been reported in asymptomatic individuals (Iqbal, 2007), following transformation of the active tachyzoites into inactive bradyzoites found in tissue cysts. A previous report has also shown that large amounts of parasite DNA can be extracted from the buffy coat compared to blood or serum (Jalal *et al*., 2004) of infected hosts.

Lower detection of *T. gondii* DNA may be caused by gene deletion. A few studies have indicated that in isolates of *T. gondii* from Uganda, the 529bp and B1 fragments have been deleted which cause the difference in the relative proportions of the repeats among the strains, while in some, the 529bp repeat element has undergone mutation (Ivović *et al*., 2012). In another report, the 529bp repeat element has been found to be absent in 4.8% of patient blood samples that were analyzed by real-time PCR and the results were false negative compared to B1 amplification (Wahab *et al*., 2010). Latest studies have questioned the quantification validity of the 529bp and B1 repeats in clinical diagnostics as the copy number of the target in *Toxoplasma* genomes have been found to be 4 to 8 and 5 to 12 times lower compared to previous estimations of 35 and 230 (Costa & Bretagne, 2012). The false negative testing samples can be determined by detection of the house keeping genes such as the SAG 2commonly used in the typing of *T. gondii* strains (Weiss & Kim 2007). However, the 529bp repeat element remains the most widely used target for molecular detection of *T. gondii* DNA (Kasper *et al*., 2009), and has been shown to have 10 to 100 times higher sensitivity compared to the B1 gene (Homan *et al*., 2000; Kong *et al*., 2012). Other conserved multi-locus sequences in the genome of *T. gondii* that may be used as targets for the detection of the parasite include ITS-1 (internal transcribed spacer) that exists in 110 copies and 18S rDNA gene sequences (Tlamçani *et al*., 2013).

Earlier studies in Tanzania have reported infection of *T. gondii* among the abattoirs and livestock keepers (Swai & Schoonman, 2009) and may indicate an occupational risk of *T. gondii* transmission. Moreover, *T. gondii* infection in humans, are usually asymptomatic and thus, may remain unrecorded (Tenter *et al*., 2000).The high infection level in chicken slaughter house workers may indicate higher *T. gondii* infection in chicken. The workers are possible regular consumers of chicken meat or chicken by products which is a possible risk of exposure to

infective bradyzoites. Chicken rearing especially the free range type is a common production system in Thika District (Ogendi *et al*., 2013), whereby the birds feed off the ground and are a good indicator of soil contamination by oocysts. Indeed chicken are increasingly being identified as important sources of *T. gondii* infection in Kenya (Dubey *et al*., 2005) and in other developing countries (Kijlstra & Jongert, 2008).

A potential risk factor for the transmission of *T. gondii* among the slaughter house workers is that, in the slaughter houses, the cattle, sheep, goats and chicken that are slaughtered may be the source of *T. gondii* infection. The workers in the slaughter houses may be exposed to *T. gondii* infection through frequent contact with animal carcasses which may contaminate the hands and the working tools such as the knives and hooks used in slaughtering. Ingestion of infective bradyzoites in tissue cysts through contaminated hands may therefore be a possible route of *T. gondii* infection to the workers and this should be further investigated.

Another possible source of *T. gondii* infection in the workers places of residence may be through ingestion of fruits and vegetables contaminated with oocysts or ingestion of undercooked meat that may be containing infective bradyzoites. Environmental contamination with infective oocysts passed by the infected cats may be another potential source of *T. gondii* infection. Nearly half of the households in Thika District keep cats and feed them with food leftovers and raw offal. Moreover, the cat owners' knowledge on proper disposal of cat feces as part of good hygiene is lacking (Ogendi *et al*., 2013). There is need to compare the *T. gondii* infection rates between the abattoir workers and people who are not involved in the slaughtering activities.

The highest prevalences of *T. gondii* infection were noted in Ruiru and Thika abattoirs. These are urban areas with mixed economic activities including keeping of livestock such as pigs, cattle, sheep goats and chicken. The two sites are also characterized with clustered dwelling places due to high population compared to Mundoro, Gachika and Gathage which have rural set ups. The risk factors for *T. gondii* transmission in the urban setting may therefore be higher compared to the rural areas due the varied sources of food that get to the markets in addition to the myriad sources of food contamination with infective oocysts from cats. The cats might be more in abundant in towns and also there is probably higher consumption of meat by the urban dwellers.

The high prevalence recorded among Ruiru and Thika slaughter house workers may also demonstrate high infection rates among the animals slaughtered and therefore the need to establish the source of the animals. Thika is among the urban set ups with the highest prevalence of HIV/AIDS (NASCOP, 2014). As a result of the high HIV prevalence in the study area, there is a higher possibility of reactivation of the parasite leading to severe outcome. In this regard, it would be important to determine the risk and prevalence of toxoplasmosis in HIV patients.

The current study also demonstrates *T. gondii* infection in baboons (*P. anubis*). Baboons scavenge food in urban settlements, may also drink water in areas possibly contaminated by humans and can be a good indicator of environmental contamination with *T. gondii*. The prevalence of *T. gondii* in baboons was within the global range of 20-50% reported in animals especially those with moderate infections (Hill & Dubey, 2014). *T. gondii* infection has been reported in the non human primates (NHPs) including the New World monkeys which have been shown to be highly susceptible to the infection (Innes, 1997). However, few surveys have been performed to detect the parasite in NHPs especially the baboons. For instance, the New World NHPs of the genera *Cebus* and *Callithrix* previously apprehended and studied by Wildlife

Screening Center (Cetas)/IBAMA, in Rio de Janeiro showed that 76.19% of the sera of *Cebus* primates tested positive for anti-*T. gondii* specific antibodies, while only 4.5% of the sera of *Callithrix,* tested positive for anti-*T. gondii* antibodies (Pires *et al*., 2012). The presence of *T. gondii* DNA in baboon serum may indicate environmental contamination with the parasite as the serum samples were obtained during quarantine period following animal capture from the wild, and therefore not exposed during quarantine. The current findings may also suggest that the baboons (*P. anubis*) may serve as a suitable NHP experimental model of toxoplasmosis. A baboon model for reproductive studies on topical microbicides for prevention of sexually transmitted infections has been developed (Obiero *et al*., 2012). The baboon model would therefore be used to understand *T. gondii* transplacental transmission that occurs in primary *T. gondii* infection and the mechanisms that lead to abortion.

The current study also compared *T. gondii* DNA detection rate between different molecular methods. Previous studies have reported that the use of PCR amplification for the detection of *T. gondii* may be unavailable in resource poor settings (Jiménez-Coello *et al*., 2012), due to the high cost of the thermocyclers and inadequate or lack of electric power supply. For this reason, the loop mediated isothermal amplification (LAMP) has been developed as an alternative to PCR method based on various targets including the 529bp repeat element (Notomi *et al*., 2000; Zhang *et al***.,** 2009; Lau *et al*., 2010; Kong *et al*., 2012).

The current study used wet LAMP targeting the 529bp multicopy element to detect *T. gondii* DNA in human serum samples. The wet LAMP assay had a lower detection rate than nested PCR. A previous study has shown that nested PCR has high sensitivity to *T. gondii* using samples with low DNA content such as the amniotic fluid (Wiengcharoen *et al*., 2004). However, in the current study, the difference in performance between the two tests was not

significant. The low sensitivity of the wet LAMP compared to nested PCR appeared to contradict the previous studies which reported that LAMP is more sensitive as compared with nested PCR (Kong *et al*., 2012), when whole-blood samples of BALB/c mice infected with *T. gondii* RH strain were analyzed by LAMP. However, the cost of nested PCR is higher than LAMP and thus LAMP could still be a better test.

The dry LAMP had a higher detection rate of *T. gondii* DNA in the human serum samples, than nested PCR assay and wet LAMP. Therefore, the dry LAMP used in this study for *T. gondii* DNA detection revealed a higher sensitivity, an observation that was in agreement with previous reports (Lau *et al*., 2010; Kong *et al*., 2012; Zhang *et al*., 2009). The higher sensitivity of freeze dried LAMP to *T. gondii* DNA compared with wet LAMP, may partly be explained by the high concentration of the parasite DNA template used. Future studies should check sensitivity of the test with lower amounts of DNA. At one instance, the detection of *T. gondii* DNA by dry LAMP showed inconsistence in sensitivity over nested PCR and wet LAMP. The failure to detect the parasites DNA may have been caused by the template copy variability or low concentration of the DNA. The study showed no significant difference in performance between dry LAMP and nested PCR and also between dry LAMP and the wet LAMP. Moreover, the lyophilization process was partially complete due to the presence of glycerol which is the antifreeze in the Bst polymerase enzyme that is available commercially for used in LAMP reactions.

Neither the wet nor dry LAMP assay amplified the DNA of the other apicomplexan parasite related to *T. gondii* (*T. b. brucei* and *T. parva*.), indicating that the test is highly specific and this is similar to the results of the other previous studies (Lau *et al*., 2010; Laohasinnarong, 2011). The dumbbell-like structure is first formed in the LAMP reaction by simultaneous amplification of the target fragment from inner (FIP and BIP) and outer (F3 and B3) primers. The Loop

primers (LF and LB) speed up the LAMP reaction by promoting formation of cauliflower-like amplification product in the reaction mixture, which appear as a ladder of multiple bands after gel electrophoresis (Notomi *et al*., 2000). The shortened detection time of between 20min and 1hour as shown with inclusion of loop primers (Zhang *et al.,* 2009), demonstrated that LAMP may be a reliable diagnostic tool, without need for further tests. The LAMP amplification product can also be detected by real-time turbidimeter. However, the use of fluorescence dyes such as SYBR green I have been reported to aid in visual inspection of the LAMP product. The LAMP assay especially using the lyophilized reagents greatly shortens the steps in the LAMP procedure and the reaction can thus be performed by the less skilled technicians (Boehme *et al*., 2007). This study shows that molecular tools (LAMP) can be used for detection of *T. gondii* in clinical samples.

5.1 CONCLUSION

- There is active *T. gondii* infection among the slaughter house workers in Thika District.
- *T. gondii* infection is also present in the baboons trapped from Thika District.
- There is no significant difference in performance between dry LAMP and nested PCR, and between dry LAMP and wet LAMP.
- The use of dry LAMP for the detection of *T. gondii* DNA greatly shortens the steps in the LAMP reaction.

5.2 RECOMMENDATIONS

1. In Thika district, there is need for public health awareness through education to control *T. gondii* among the slaughter house workers.
2. A study to examine whether the prevalences of *T. gondii* are higher or lower among other residents (non abattoir workers) in the study area needs to be done.
3. The baboon (*P. anubis*) can be used as an experimental model in the investigation of transmission, pathophysiology, diagnosis and treatment of *T. gondii* infection.
4. The dry LAMP may be used for the detection of *T. gondii* in clinical samples. However further evaluation on the lyophilized reagent storage temperature condition and the duration of stability should be studied.

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APPENDIX 1. Ethical review letter from Kenya Medical Research Institute (KEMRI)

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **December 18, 2012**

TO: **MR. JAMES MAINA ICHAGICHU (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. SAMUEL KARIUKI,**
DIRECTOR, CMR,
NAIROBI

forwarded. 20/12/12.

Dear Sir,

RE: **SSC PROTOCOL No. 2354 – REVISION 2 (*RE-SUBMISSION*): EPIDEMIOLOGY OF TOXOPLASMOSIS AND IDENTIFICATION OF PARASITE GENOTYPES INFECTING HUMANS IN THIKA DISTRICT, KENYA**
(*VERSION 1.1 DATED 7TH DECEMBER 2012*)

Reference is made to your letter dated December 7, 2012. The ERC Secretariat acknowledges receipt of the revised proposal on December 7, 2012.

This is to inform you that the Committee determines that the issues raised at the 209th ERC meeting of 30th October 2012 and on 12th November 2012 are adequately addressed. Consequently, the study is granted approval for implementation effective this **18th day of December 2012** for a period of one year.

Please note that authorization to conduct this study will automatically expire on **December 17, 2013**. If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **November 5, 2013**. The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

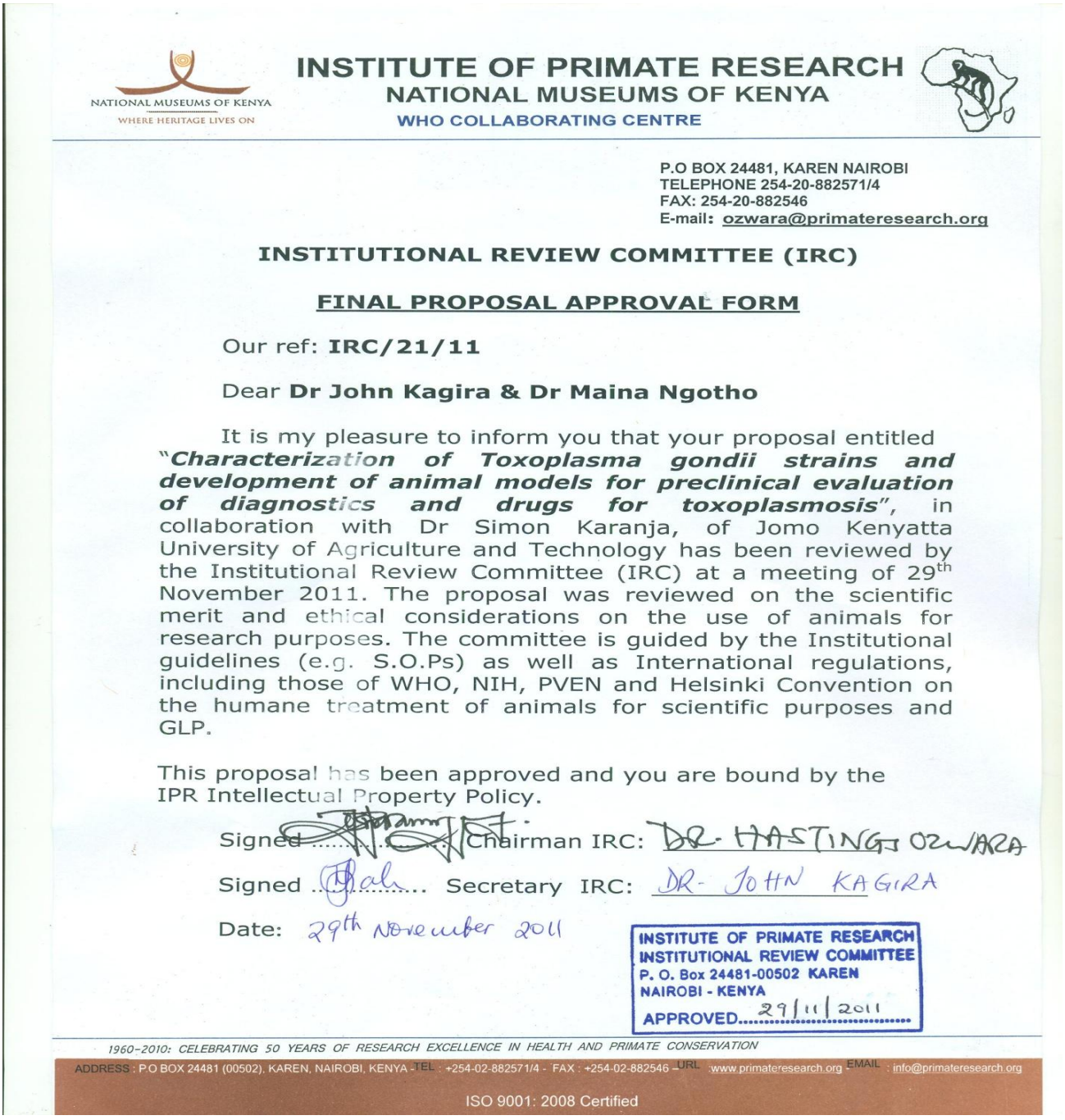
You are required to submit any proposed changes to this study to the SSC and ERC for review and the changes should not be initiated until written approval from the ERC is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the ERC and you should advise the ERC when the study is completed or discontinued.

Work on this project may begin.

Sincerely,

DR. CHRISTINE WASUNNA,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

APPENDIX 2. Ethical review letter from Institute of Primate Research (IPR)



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INSTITUTE OF PRIMATE RESEARCH
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INSTITUTIONAL REVIEW COMMITTEE (IRC)

FINAL PROPOSAL APPROVAL FORM

Our ref: **IRC/21/11**

Dear **Dr John Kagira & Dr Maina Ngotho**

It is my pleasure to inform you that your proposal entitled "***Characterization of Toxoplasma gondii strains and development of animal models for preclinical evaluation of diagnostics and drugs for toxoplasmosis***", in collaboration with Dr Simon Karanja, of Jomo Kenyatta University of Agriculture and Technology has been reviewed by the Institutional Review Committee (IRC) at a meeting of 29th November 2011. The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines (e.g. S.O.Ps) as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

This proposal has been approved and you are bound by the IPR Intellectual Property Policy.

Signed ..... Chairman IRC: DR. HASTINGS OZWARA

Signed ..... Secretary IRC: DR. JOHN KAGIRA

Date: 29th November 2011

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
P. O. Box 24481-00502 KAREN
NAIROBI - KENYA
APPROVED 29/11/2011