DEVELOPMENT AND EVALUATION OF A LOOP MEDIATED ISOTHERMAL AMPLIFICATION TECHNIQUE FOR THE DETECTION OF HOOKWORM INFECTION IN FEACAL SAMPLES

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Development and evaluation of a loop mediated isothermal amplification technique for the detection of hookworm infection in feacal 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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Robert Mugambi Muriuki

This thesis has been submitted for examination with our approval as University Supervisors.

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JKUAT, Kenya

DEDICATION

This thesis is dedicated to the entire Muriuki family for their overwhelming support and prayers and thanking God for allowing and giving me the strength and ability to carry out this research.

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ABBREVIATIONS AND ACRONYMS

ALB	Albendazole
B3	Backward outer primer
BIP	Backward Inner Primer
BLP	Backward loop primer
Bst	*Bacillus stearothermophilus*
DNA	Deoxyribose nucleic acid
dNTPs	Deoxy nucleotide triphosphates
F3	Forward outer primer
FECT	Formalin-ether concentration technique
FG	Femtogram
FIP	Forward Inner Primer
FLP	Forward loop primer
ITS 1 & 2	Internal transcribed spacer 1 & 2
LAMP	Loop mediated isothermal amplification
NTDs	Neglected Tropical Diseases
PCR	Polymerase chain Reaction
RPM	Revolutions per minute
SAF	Sodium acetate-acetic acid
STH	Soil transmitted Helminths
WHO	World Health Organization

ABSTRACT

Hookworm infection is a major concern in sub-Saharan Africa, particularly in children and pregnant women. *Necator americanus* and *Ancylostoma duodenale* are responsible for this condition. Accurate diagnosis is essential for the formulation of effective control measures. Currently most research conducted on epidemiology of hookworms has relied on the use of conventional microscopy for identification of parasite ova in faeces. The benefits are mainly technical simplicity and low cost; however, these techniques are limited to the fact that most of nematode eggs are morphologically indistinguishable from those of other species, they also lack the sensitivity to detect low level infections and thus cannot be effective in areas where mass chemotherapy is employed as a control strategy against hookworms and other soil transmitted helminths. Over-reliance on this microscopy based techniques that is Kato-Katz that is prone to human errors and leads to in most cases misdiagnosis and poor implemented control strategies. With that in mind it is of great importance to develop efficient molecular diagnostic tools, which have proven to be more sensitive in detecting low level infections with reliability and reproducibility and specific enough to differentiate between infections by different species. In this study, I aimed to employ a Loop mediated isothermal amplification to diagnose hookworm infection, specifically *N. americanus* from faecal samples. This LAMP assay was based on internal transcribed spacer 2 (ITS-2) of nuclear ribosomal DNA (rDNA) because of its low mutation rates and ability to differentiate between species. The assay employed four primers; two inner primers and two outer primers designed by an online based program, Primer explorer version 4. *Bst* DNA polymerase containing the 5'-3' polymerase and lacking 5'-3' exonuclease activity with high displacement activity was used. This technique showed to be 100 % specific when used in detecting DNA from other commonly occurring soil transmitted helminths, that is*, S. mansoni, A. lumbricoides* and *T. trichiura* and 97% sensitive with a detection limit as low as 0.4FG. When compared to Kato-Katz, LAMP proved more robust by detecting three samples negatives, which were diagnosed as positive by Kato-Katz, highlighting

the limitation that Kato-Katz readings are subject to human errors non-random distribution of eggs in stool and day to day variation in egg output. In terms of agreement, between the two techniques, there was an excellent agreement level with a kappa coefficient of 0.9. The LAMP assay has demonstrated that it can be utilized as an appropriate diagnostic method for the detection of *N. americanus* DNA in human faecal samples because of its high sensitivity, specificity. It holds great promise as a useful tool for use in disease control where infection intensities have been significantly reduced.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Hookworms are nematodes belonging to the family *Ancylostomatidae*, a part of the Superfamily *Strongyloidea*. They are blood feeding intestinal worms that infect about 1 billion people globally, causing approximately 135,000 deaths per year (Ngui, Lim, & Chua, 2012). Human hookworm infection is of major public health significance particularly in children and pregnant women due to chronic intestinal blood loss which may result in iron-deficiency, anaemia and hypoalbuminemia (Crompton, 2001). Most of the infections in humans are caused by two common species, *Necator americanus* and *Ancylostoma duodenale* (Chan, Medley, Jamison, & Bundy, 1994).

A. duodenale is usually a lone species in temperate climates and has spotty distribution in the tropics. It is mainly restricted to the Middle East, Northern Africa, India, Australia and Europe. On the other hand, *N. americanus* is primarily a parasite of the tropical regions of the world. It is mainly found in Sub-Saharan Africa, Eastern Asia and South East Asia (de Silva et al., 2003). Furthermore, zoonotic infections with canine and/or feline hookworm such as *A. ceylanicum*, *A. caninum*, and *A. braziliense* have also been reported in many parts of the world. Recently, zoonotic *Ancylostomiasis* caused by *A. ceylanicum* has been occasionally reported in rural communities in Malaysia (Ngui *et al*., 2012), Thailand (Jiraanankul *et al*., 2011) and Laos PDR (Sato *et al*., 2010).

Sub Saharan Africa bears the greatest hookworm burden, which is a leading cause of anaemia infecting, 40 to 50 million school going children and 7 million pregnant women, (Hotez & Kamath, 2009).

The greatest number of hookworm infection cases occur in Nigeria (38 million) and the Democratic Republic of Congo (DRC, 31 million), followed by Angola, Ethiopia, and

Cote d'Ivoire (10–11 million) (Molyneux *et al*., 2005). Coastal regions and areas of extremely high temperatures (where land surface temperatures exceed 37°C-40°C) are areas of notable high hookworm prevalence and intensity as compared to other helminth infections (Mabaso *et al*., 2004). In Kenya the hookworm prevalence is at 28.7% with majority of these infections occurring at the coastal region (Hotez & Kamath, 2009).

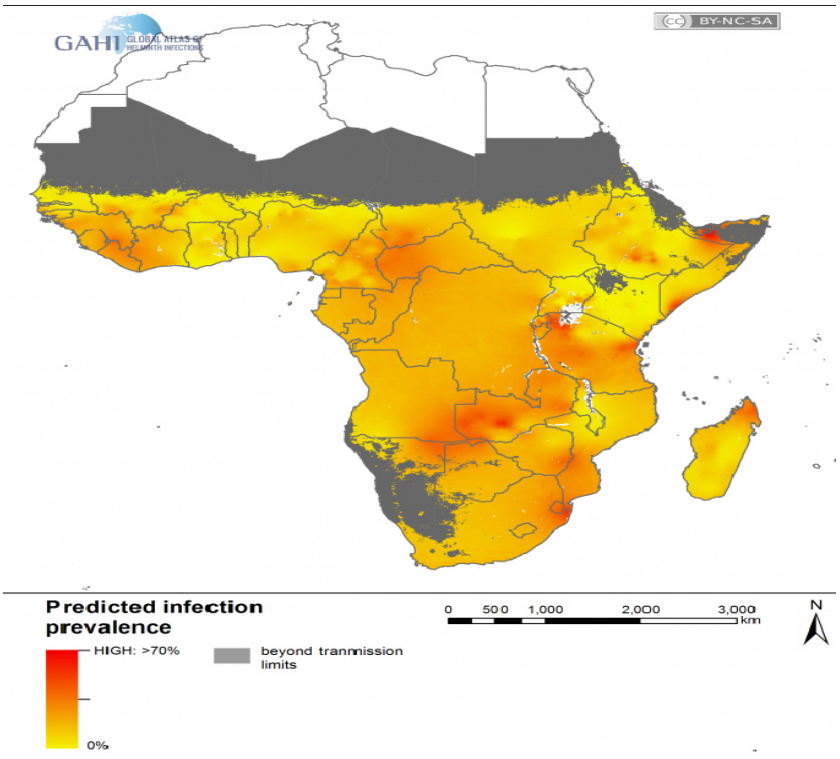


Figure 1.1: Prevalence of hookworm in Africa.

Adapted from GAHI, Global Atlas of helminth infection (www.thiswormyworld.org)

1.2 Life cycle and Transmission of Hookworms

Hookworm has a direct lifecycle. The infective stage is a third stage larva that penetrates the skin upon contact with contaminated soil. While in the soil the third stage larvae are in a state of developmental halt; maturity continues after the larvae enter the human host (Brooker *et al*., 2004).

Activated larvae migrates through the vasculature and are swept via the afferent circulation to the right side of the heart and then to the pulmonary vasculature. The larvae break out of lung capillaries and into the lung parenchyma where they ascend the alveoli, bronchioles, bronchi and trachea when they are coughed and swallowed. The third stage larvae enter the gastrointestinal tract where they moult twice and develop to the adult stage (Hotez *et al*., 2003).

It takes about 5-8 weeks from the time the third stage larvae infects humans until they reach sexual maturity and mate. Each female hookworm produces thousands of eggs daily. Intestinal blood loss begins just prior to egg production and deposition, and continues for the life of the hookworm (Hotez *et al*., 2003).

The eggs are transparent, thin-shelled and ovoid, with blunt, rounded ends, measuring approximately 60 µm × 40 µm. Hookworm eggs exit the body in faeces. They hatch after a day or two (24-48hrs), when deposited in soil with adequate warmth, shade and moisture, into first stage larvae (L1) that feed on organic debris and bacteria present in the soil. The first stage larvae, moults twice and develops into the infective stage, the third stage larvae (L3). The third stage larvae can live in the soil for several weeks until they exhaust their lipid metabolic reserves. The major clinical manifestation of hookworm infection is the consequences of chronic intestinal blood loss resulting in iron-deficiency leading to anaemia. Hypoalbuminemia develops when blood loss exceeds the intake and reserves of host iron and protein although the degree of iron-deficiency anaemia induced by hookworms depends on the species; *A. duodenale* causes greater blood loss than infection with *N. americanus* (Hotez *et al*., 2003).

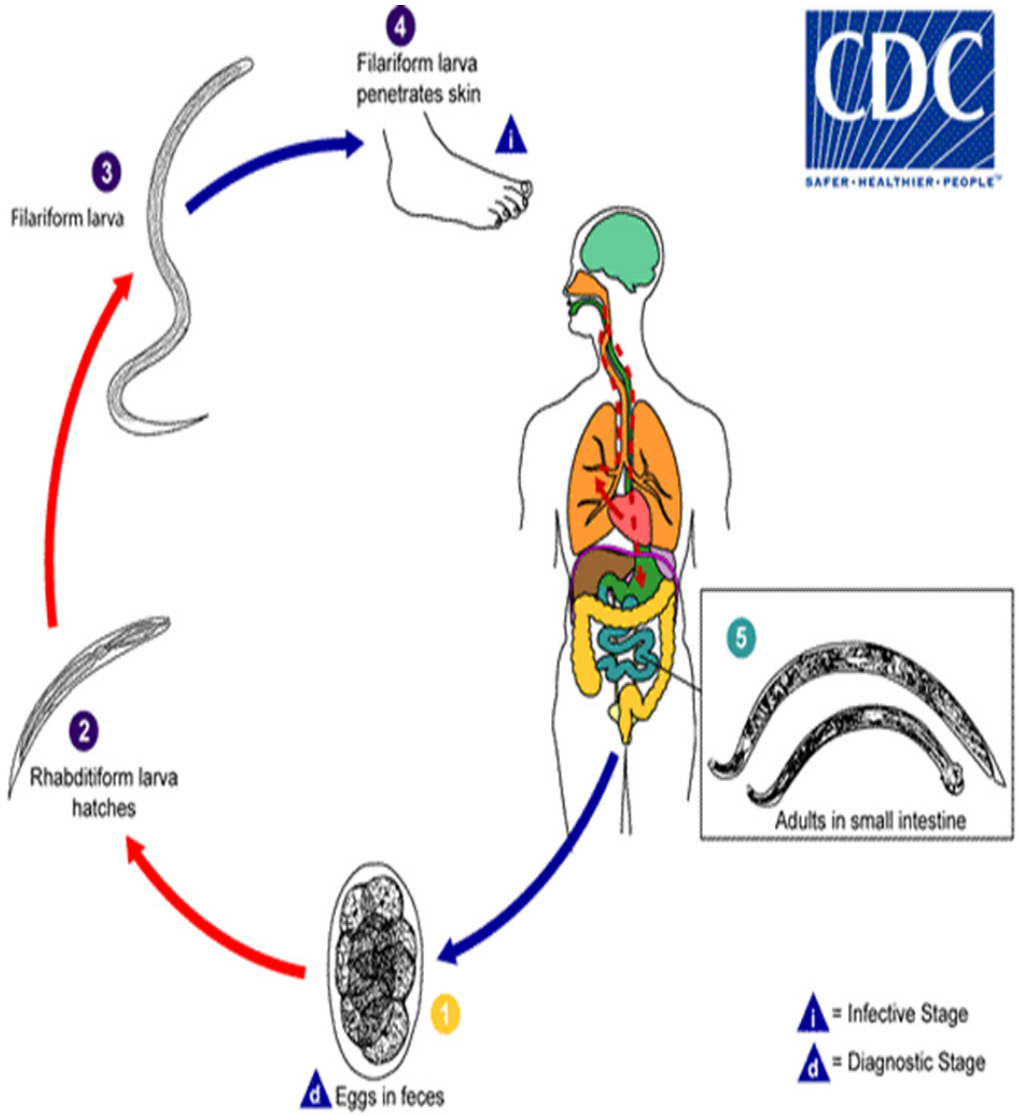


Figure 1.2: Life cycle of hookworm.

Adapted from CDC, Centre for Disease Control and Prevention, (www.cdc.gov/parasites/hookworm/biology.html).

1.3 Current efforts to reduce Morbidity

Per the World Health Organization (WHO), infection intensities are defined as either light, moderate or heavy infections. Light infections are defined as those with 1-1,999 eggs per gram. Moderate infection are defined as those with 2,000–3,999 eggs per gram

(epg) of stool and heavy infections as those with 4,000 epg or more (Hotez *et al*., 2005). The World health organization (WHO) recommended method of controlling hookworm infections is through mass drug administration by administration of anthelmintic, to the infected population with the objective of reducing infection intensity and transmission potential. This strategy has mostly lead to reduced morbidity and mortality associated with the disease. The increase of chemotherapy programs is underway in various parts of Africa, Asia and South America, mainly targeting school going children (Albonico *et al*., 2008).

1.4 Treatment and diagnosis of hookworm infection

The most widely used drugs in the treatment of hookworms are the Benzimidazole drugs, that is, Albendazole (ALB) and Mebendazole. While both drugs show broad-spectrum anthelminthic activity, a single dose of Albendazole is more effective than Mebendazole in the treatment of hookworms (Bennett & Guyatt, 2000).

Diagnosis of hookworms lies mainly on the detection of hookworm eggs in human feacal samples. There are several techniques that have been employed in the diagnosis of hookworms; these techniques are Kato-Katz, ether-concentration, FLOTAC, immunological techniques and molecular techniques like polymerase chain reaction (PCR) (Glinz *et al*., 2010).

1.4.1 Kato-Katz

Currently Kato-Katz technique is the most widely used copro-microscopic method. It is based on the principle that people infected with soil transmitted helminths pass the eggs of the worms through their faeces. By examining a stool sample under a microscope it is possible to count the number and the type of eggs that are present (World health Organization, 2008).

This technique is widely used due to its simplicity and low cost. However, this technique has drawbacks; it is limited by day to day variation in helminth egg excretion, requires well trained and experienced laboratory technician for accurate identification of parasites (Tarafder *et al*., 2010), it also lacks sensitivity, if only a single stool sample is examined particularly in areas with high proportions of light-intensity infection (Knopp *et al*., 2008). Moreover, it is not sensitive enough to differentiate between the eggs of *N. americanus* and those of *A. duodenale* (Gasser *et al*., 2008).

1.4.2 Formol Ether Concentration

Formol ether concentration is another method commonly used in the diagnosis of hookworm and other helminthic infections. Formol ether concentration is a sedimentation method that uses centrifugation to concentrate the helminth ova at the bottom of the tube; ether is used as an extractor of debris and fat from the faeces (Steinmann *et al*., 2010). The formalin-ether concentration technique (FECT) is a widely used sedimentation technique for the diagnosis of intestinal protozoa in preserved stool samples (Becker *et al*., 2011). It allows the concurrent diagnosis of intestinal protozoa, and is sometimes used in combination with the Kato-Katz method to enhance diagnostic sensitivity for helminths (Steinmann *et al*., 2010). An important quality of FECT is that is uses preserved stool samples fixed in sodium acetate or diluted formalin and thus allows sample storage and analysis at later time points (Glinz *et al*., 2010). This technique however, has one major drawback when it comes to hookworm diagnosis. A study done by (Glinz *et al.*, 2010) revealed a decrease in feacal egg count as a function of preservation time using sodium acetate-acetic acid formalin (SAF), suggesting a negative impact of this preservation medium on hookworm eggs and this its diagnosis.

1.4.3 FLOTAC

FLOTAC is a recent technique that holds promise in the diagnosis of soil transmitted helminths Cringoli *et al*., 2010). The FLOTAC technique takes advantage of the fact that

during flotation, parasitic elements such as helminth eggs gather in the apical portion of the flotation column and can be readily transferred to a slide for subsequent viewing under a microscope (Glinz *et al.*, 2010), this allows the parasitic elements to be separated from feacal debris and thus facilitating their identification and quantification. This technique allows a 24-fold higher amount of stool to be examined than a single Kato-Katz thus proving more sensitive than Kato-Katz (Cringoli *et al*., 2010). FLOTAC is also of low cost and thus is good for resource-limited settings, but it is time consuming and laborious.

1.4.4 Serological techniques

Serological techniques are also available for the diagnosis of hookworm (Bungiro & Cappello, 2005). These techniques are based on the detection of specific *invitro* antigen-antibody reactions. These methods are of high sensitivity and are predicted most likely to reflect active infection (Coulibaly *et al*.,2013). However these methods are not able to achieve the differentiation between current and recent past hookworm infections, and usually suffer from problems of cross-reactivity (Gasser, 2006). They are also difficult in evaluating results especially when a patient originates from areas where the infection is endemic (Bruschi & Castagna, 2004).

1.4.5 Molecular techniques

Molecular techniques have been applied in the diagnosis of hookworms. Polymerase chain reaction based techniques have been developed for the detection of hookworm infections (Wang *et al*., 2012). These molecular techniques offer great advantages since they are more sensitive and more specific than the traditional microscopic techniques (Gasser *et al*., 2008). Their main drawback is that they are costly, require expensive equipment and are thus not applicable in resource-poor settings.

Recently, a novel method of diagnosis, loop mediated isothermal amplification (LAMP) was developed by Notomi *et al*. (Notomi *et al*., 2000). This technique is based on the

principle of auto-cyclic strand displacement reaction being performed at a constant temperature using a DNA polymerase with high displacement activity (Parida *et al*., 2008). The technique has been shown to amplify DNA with high specificity, efficiency and rapidity under isothermal conditions (Notomi *et al*., 2000). Moreover, LAMP is easy to carry out and doesn't require expensive equipment as dictated by other DNA based diagnostic techniques. This makes LAMP an ideal tool for field based diagnosis (Mori *et al*., 2006).

1.5 Problem Statement

Hookworms are blood feeding intestinal nematodes that infect almost 600 million people worldwide, resulting in up to 135,000 deaths annually (Hotez & Kamath, 2009). It is also one of the most important in terms of disease burden, accounting for up to one-third of the total burden from NTDs in Sub Saharan Africa (Bethony *et al*., 2006). In Kenya, the hookworm prevalence is 16.9 % (Kepha *et al*., 2015). *Necator americanus* and *Ancylostoma duodenale* are two most common species causing infection in humans. In general, mixed infections of these hookworms are common in many endemic areas especially among people in tropical and subtropical countries with low socioeconomic status. In terms of disability-adjusted life years, hookworm infection is the second most important parasitic infection after Malaria (Jia *et al*., 2012). With the current WHO recommended approach to the control of STH being Mass Drug Administration, low infection intensity will be common. Current parasitological techniques are not sensitive at detecting low infection, are time consuming and rely heavily on human reading and are prone to error.

The current study aims at developing a sensitive, high throughput molecular technique to detect *N. americanus,* which is the prevalent of the two human hookworm infections in Sub Saharan Africa. This technique should be able to pick up low intensity infections and will thus translate to better treatment of hookworm infection, improved control strategies and greatly reduce disease morbidity.

1.6 Justification

Diagnosis of helminths is achieved through standard parasitological tests such as Kato-Katz and formal ether concentration. Although these methods are direct and specific, they miss on less intense infection. The World Health Organization has put in place strategies of reducing infection intensity and morbidity by way of mass drug administration. With the anticipated lowering of infection intensity following intervention, there is need for a more sensitive and affordable diagnostic technique with high throughput potential that can be applied at point of care. Simple, cheap, sensitive and specific assays for routine diagnosis of helminth infection are not yet available (Doenhoff *et al*., 2004). There is a need for the development of more specific and sensitive molecular based diagnostic techniques that will improve on the traditional microscopy based techniques. The LAMP technology-based diagnostic assays promise to revolutionize disease diagnosis as tools for point of contact/care diagnosis. With a more sensitive diagnostic kit, there will be great improvements in detections of infection cases, reduced morbidity and better informed control strategies.

1.7 Research Questions

1. Which DNA target in hookworm parasites with markers is suitable for design of appropriate primers for LAMP assay?
2. How is LAMP assay optimized for detection of hookworm infection?
3. How does LAMP technique compare to Kato-Katz procedure targeting feacal samples?

1.8 Null Hypothesis

LAMP technique and other parasitological diagnostic procedures have no difference in their detection of hookworm infections in feacal samples.

1.9 General Objective

To develop a loop-mediated isothermal amplification (LAMP) tool for the detection of hookworm infections in feacal samples.

1.9.1 Specific objectives

1. To identify a DNA target in hookworm parasites suitable as a marker for loop mediated isothermal amplification (LAMP) assay.
2. Optimize a LAMP assay for detection of hookworm infection (*Necator americanus*).
3. To evaluate the LAMP technique in comparison with parasitological diagnostic procedures using feacal samples.

CHAPTER TWO

LITERATURE REVIEW

2.1 Loop mediated Isothermal Amplification (LAMP)

Loop mediated isothermal amplification is a novel gene amplification technique developed by Notomi *et al.* (Notomi *et al*., 2000) that amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. According to (Notomi *et al*., 2000) LAMP can amplify a few copies of DNA to 10^9 in less than an hour under isothermal conditions and with greater specificity.

This method employs a DNA polymerase and a set of four to six primers that recognize a total of six to eight distinct sequences on the target DNA. The primers in use are; two outer primers F3 (forward outer primer) and B3 (backward outer primer), two inner primers FIP (Forward inner primer) and BIP (backward inner primer) and two loop primers FLP (forward loop primer) and BLP (backward loop primer). An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP.

The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem–loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem–loop DNA and a new stem–loop DNA with a stem twice as long. The final products are stem–loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity (Notomi *et al*., 2000). This nucleic

acid amplification technique has emerged as a simple rapid diagnostic tool for the early detection and identification of microbial diseases (Parida *et al*., 2008).

Bst polymerase is a thermostable DNA Pol 1 that was isolated in 1968 (Stenesh *et al*., 1968) from the thermophilic bacterium *Bacillus stearothermophilus (Bst)* which proliferates between 39°C and 70°C. *Bst* Pol I is active at an optimal temperature of 65°C, and is inactivated after 15 min incubation at 75°C. This enzyme contains 5'→ 3' polymerase activity but lacks 5'→3' exonuclease activity and requires high Mg^{2+} concentration for maximum activity. Protease digestion of *Bst* DNA Pol 1 generates two protein fragments. The large protein fragment of *Bst* DNA Pol 1 is thermostable, and thus very useful for sequencing reactions performed at 65°C to avoid problems due to hairpin formation. The Large Fragment of *Bst* DNA Pol I show a faster strand displacement than its full-length counterpart thus making *Bst* enzyme effective for amplification of DNA based targets. It has a disadvantage in that it cannot amplify RNA without the addition of a reverse transcriptase for conversion of RNA template to cDNA that serves as a target for LAMP.

2.1.1 Principle of loop mediated isothermal amplification

The chemistry of loop mediated isothermal amplification is based on the principle of auto-cyclic strand displacement reaction being carried out at a constant temperature using a DNA polymerase. There are two steps of LAMP amplification consisting of non-cyclic and cyclic steps (Ushikubo, 2004)

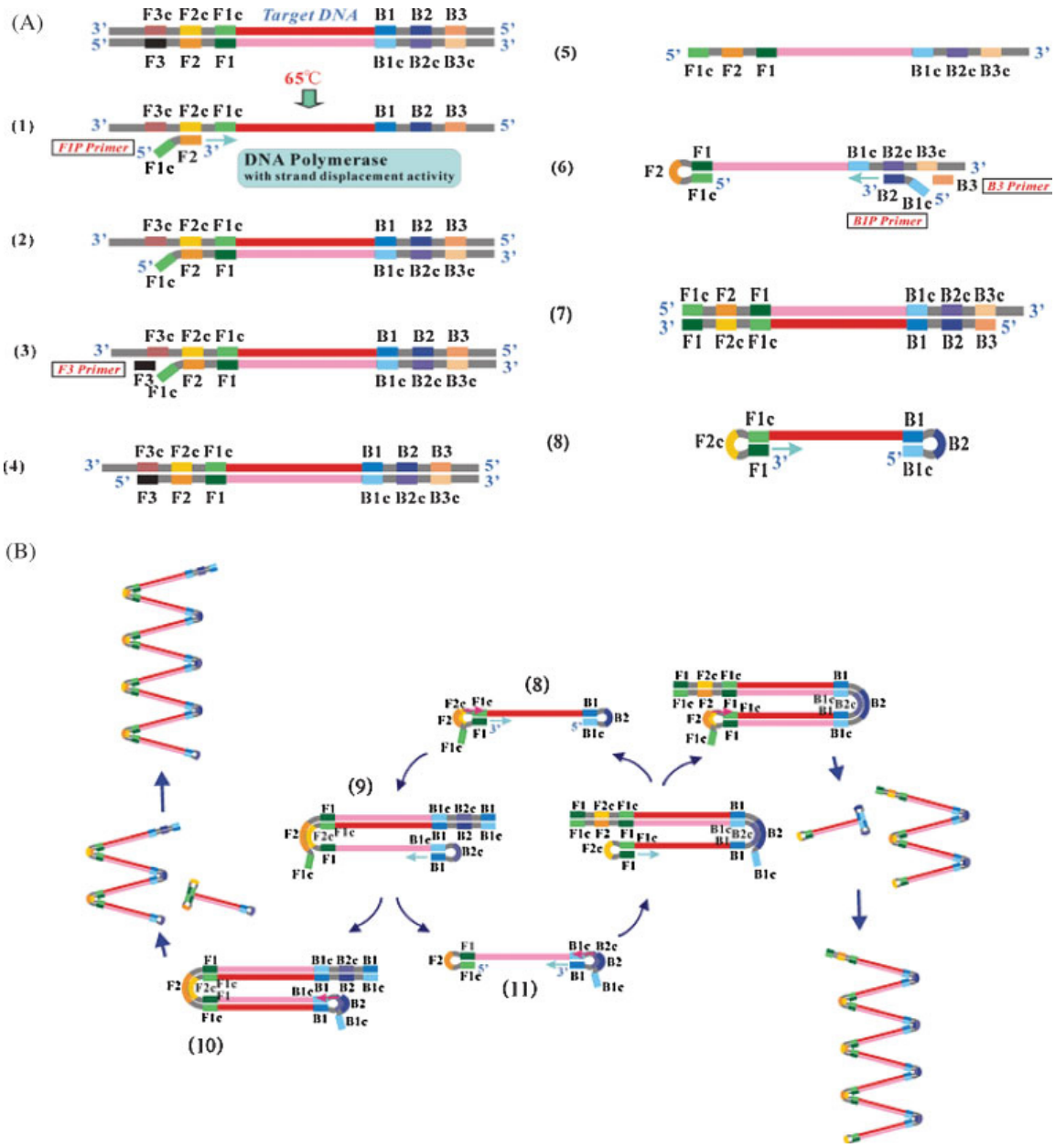


Figure 2.1: Illustration of the LAMP principle.

The illustration consists of two steps, Non-cyclic (a) cyclic amplification (b) Ushikubo, 2004.

2.1.1.1 Non-cyclic step

In this step, there is the formation of DNA with stem-loop at each end that serves as the starting structure for the amplification by LAMP cycling. Double stranded DNA is in the condition of dynamic equilibrium at temperatures of about 65°C. One of the LAMP primers can anneal to the complementary sequence of the double stranded target DNA, then initiate synthesis using the DNA polymerase, in specific *Bst* polymerase, with high strand displacement activity displacing and releasing a single stranded DNA (Ushikubo, 2004). In this amplification technique, there is no need for heat denaturation of the double stranded DNA into single strand. Through the activity of *Bst* polymerase with strand displacement activity a DNA strand complementary to the template DNA is synthesized starting from the 3' end of the F2 region of the FIP. The F3 primer anneals to the F3c region outside the FIP, on the target DNA and initiates strand displacement DNA synthesis, releasing the FIP-linked complementary strand (Ushikubo, 2004) . A double strand is formed from the DNA strand synthesized from the F3 primer and the template DNA strand. The FIP-linked complementary strand is released as a single strand because of the displacement by the DNA strand synthesized from the F3 primer. The released single strand forms a stem-loop structure at the 5' end because of the complementary F1c and the F1 regions. This single stranded DNA acts as a template for the BIP initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis. The BIP anneals to the DNA strand produced in the above step. Synthesis of the complementary DNA starts from the 3'end of the BIP. Through this process the DNA reverts from a loop structure to a linear structure. B3 primer anneals to the outside of the BIP and then through the activity of Bst polymerase and starting at the 3'end, the DNA synthesized from the BIP is displaced and released as a single strand before DNA synthesis from the B3 primer. The BIP-linked complementary strand displaced forms a dumbbell structure (Ushikubo, 2004). This structure is quickly converted into a stem-loop DNA by self-primed DNA synthesis and serves as a starting structure for the

exponential amplification (Parida *et al*., 2008). The processes in this step are illustrated in figure 2.1 (A).

2.1.1.2 Cyclic Amplification

In subsequent LAMP cycling (figure 2.1 B) one internal primer hybridizes to the loop on the product and initiates displacement DNA synthesis yielding the original stem-loop DNA with a new stem-loop DNA with a stem twice as long. FIP anneals to the single stranded region in the stem loop DNA and primes strand displacement DNA synthesis, releasing the previously synthesized strand. This released strand forms a stem-loop at the 3' end because of the complementary B1 and the B1c regions (Ushikubo, 2004). Starting from the 3'end of the B1 region, DNA synthesis starts using self-structure as a template and releases FIP linked complementary strand. The released single strand then forms a dumbbell-like structure as both ends have complementary F1-F1c and B1-B1c regions respectively. Furthermore, BIP anneals to the B2c region and primes strand displacement DNA synthesis, releasing the B1-primed DNA strand. Because of this process, various sized structures consisting of alternately inverted repeats of the target sequence on the same strand are formed. The cycling reaction continues leading to accumulation of 10^9 copies of the target in less than an hour. The final products are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the targets in the same strand (Parida *et al*., 2008). LAMP amplification can also be accomplished with the addition of two loop primers in addition to the two inner and two outer primers. The use of loop primers reduces the amplification time (Nagamine *et al.,*2002).

2.1.2 LAMP reagents

LAMP reagents vary in some major aspects to one of the techniques more widely used, conventional PCR. The use of six primers provide a greater level of target specificity

than the one achieved with two primers normally used in PCR (Notomi *et al*., 2000) as well as the use of a DNA polymerase with strand displacement activity (*Bst* DNA polymerase) under isothermal conditions and lower temperature (60-65°C). *Bst* DNA polymerase contains the 5´→3´ polymerase activity, but lacks 5´→3´ exonuclease activity (New England Bio labs, Beverly, MA). The enzyme has a strand-displacement type DNA polymerase activity, which synthesizes a new DNA strand while separating the hydrogen bond of the double stranded template DNA. Since the strand-displacement DNA polymerase does not require heat dissociation of double-stranded DNA, the DNA can be synthesized at a constant temperature.

Optimum temperature for *Bst* DNA polymerase ranges from 50°C -65°C, which facilitates primer annealing and enhances tolerance to inhibitors typically found in diagnostic samples; an advantage compared to other polymerases. However, inhibition above 65°C and inefficient incorporation of dNTPs has been reported (Tanner, 2015). The DNA polymerase is supplied with thermopol buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton®-X-100, pH 8.8 at 25°C) that ensures polymerase activity (New England Bio labs, Beverly, MA).

The concentration of dNTPs in the PCR reaction mixture also plays an important role in achieving the desired results. Slight deviation from the required concentration has led to dramatic increase of disincorporation levels of dNTPs (McPherson and Moller, 2000). In most of the publications about LAMP, dNTPs concentration is very high (1.4 mM) compared to conventional PCR (Li *et al*., 2011). The presence of magnesium source ($MgSO_4$) in conventional PCR is required for polymerase activity, primer annealing, strand dissociation and product specificity. Previous reports on conventional PCR have demonstrated that optimum concentrations of MgSO4 should contain 0.5 to 2.5 mM over the total dNTPs concentration (Innis *et al.*, 1990). Previous LAMP procedures concentrations up to 2.5 mM have been reported (Ravindran *et al.*, 2012). In LAMP assays, magnesium concentration can also interfere with dye reactions (Das *et al*., 2012).

Previous studies have reported that the addition of enhancers such as Betaine improves yield and specificity in GC rich regions and decreases the production of undesired products. DNA regions rich in GC concentration can sometimes be problematic due to inefficient separation of DNA strands or the formation of secondary structures. Betaine decreases the amount of energy necessary to separate DNA strands (Jensen *et al*., 2010).

2.1.3 Primer Design

Designing of a highly sensitive and specific primer set is essential for performing LAMP assay. LAMP primers can be designed a web-based software known as primer Explorer and can be accessed at primer explorer (https://primerexplorer.jp/e/). The LAMP primers are based on six regions in the target sequence. The primers are designated as FIP (forward inner primer), BIP (backward inner primer), F3 (forward outer primer), B3 (Backward inner primer), FLP (forward loop primer) and finally BLP (backward loop primer) (Notomi *et al*., 2000). The loop primers serve the function of accelerating the rate of reaction (Nagamine *et al*., 2002). There are several factors to consider when designing LAMP primers. The factors are Tm (melting temperature), stability at the end of each primer, GC content and secondary structure; the melting temperature is estimated using the nearest–neighbour method. This method is currently considered the best approximation method that gives the value closest to the actual value. The calculated Tm is affected by experimental conditions such as salt concentration and primer concentration, thus it is advisable to calculate the Tm under fixed experimental conditions, that is oligo concentration at 0.1 μM, sodium ion concentration at 50 mM, magnesium ion concentration at 4 mM (Eiken Chemical Company Limited, 2013). The melting temperature for each region is designed to be about 65°C (64 - 66°C) for F1c and B1c, about 60°C (59 - 61°C) for F2, B2, F3, and B3, and about 65°C (64 - 66°C) for the loop primers.

The end of the primers serves as the starting point of the DNA synthesis and thus must have certain degree of stability. The primers are designed so that the free energy is -

4kcal/mol or less (Eiken Chemical Company Limited, 2013). Primers are designed so that their GC content is about 40% to 65%. Primers with GC content between 50% and 60% tend to give relatively good primers (Eiken Chemical Company Limited, 2013). It is important, particularly for the Inner primer, that primers are designed so that they do not form secondary structures. To prevent the formation of primer dimers, it is also important to ensure that the 3' ends are not complementary (Eiken Chemical Company Limited, 2013).

2.1.3 Application of LAMP in other parasitic species

Although the inception of LAMP refers to 2000, it became popular only after 2003**.** Since then LAMP has been used in clinical diagnosis of various parasites. Abbasi *et al*., 2010, developed a technique that can detect infected snails from early prepatency, that is carried out at 63°C in a water bath for two (2) hours and amplicons are viewed using SYBR green I stain. The primers used were developed from sm1-7 of *S. mansoni.* The assay developed has a detection sensitivity of 0.1fg and it was possible to detect infected snails from the first day of snail exposure to miracidia when the assay was applied for examining infected laboratory snails. The developed assay was to facilitate large scale evaluation of post-intervention transmission potential.

A study conducted by Poon (2005) developed a sensitive and inexpensive malaria diagnosis test for *Plasmodium falciparum* directly from heat-treated blood and results were interpreted by a turbidity meter in real time. The primers were designed to detect the highly conserved 18s ribosomal RNA gene of *P. falciparum*. The reaction was conducted at 60°C for 120 minutes in a real-time turbidity meter. Loop mediated isothermal amplification has also been developed in detection of *Schistosoma japonicum* DNA in feacal and serum samples by Xu *et al.,* 2010. The LAMP assay was based on the highly repetitive retrotransposon SjR2 and had a detection limit of 0.08fg which proved to be 10^4 more sensitive than the conventional polymerase chain reaction.

Nkouawa *et al.* (2009) developed a LAMP technique that detects *Taenia* species in feacal specimens and compared it to multiplex PCR, finding out that LAMP was more sensitive than multiplex PCR, 88.4% to 37.2% respectively. In this study, it was noted that LAMP has several advantages over multiplex PCR method of diagnosis, 1). LAMP is more sensitive, easier, than the multiplex PCR method 2). It is possible to detect a positive LAMP reaction from a negative by visual endpoint judgment of the turbidity caused by the production of magnesium pyrophosphate as a by-product of the reaction. 3). The LAMP method performed within 90 minutes is faster than the multiplex PCR, which takes at least 4 hours for the completion of the two-round polymerase chain reaction 4). LAMP is inexpensive since the method is carried out using a simple incubator such as a water bath or a heat-block unlike a thermocycler in PCR. Liu *et al.,* (2011) developed a LAMP assay that detects specifically *Angiostrongylus cantonensis* in *Achatina fulica* which is the intermediate host of the *A. cantonensis*. Primers were designed based on the internal transcribed spacer 1 (ITS-1) of the ribosomal DNA. Compared to polymerase chain reaction, LAMP once again showed that it was 10 times more sensitive.

Kuboki *et al*. (2003) developed a LAMP assay for the detection of parasites in the *Trypanosoma brucei* group, (*T. brucei brucei, T. brucei gambiense, T. brucei rhodiesiense and T. evansi* and *T. congolense).* The study showed that sensitivity of the LAMP based technique was up to 10 times higher than that of PCR based methods. *In vivo* studies in mice infected with human-infective *T. brucei gambiense* highlighted the potential clinical importance of LAMP as a diagnostic tool for the identification of African trypanosomiasis.

2.1.4 Detection of LAMP products

2.1.4.1 Turbidity

Initially, turbidity of the final product was used to detect positive amplification of DNA (Notomi *et al*., 2000; Yoshida *et al*., 2005and Parida *et al*., 2008). (Parida *et al*., 2008) reported that to observe the turbidity in the form of a white precipitate, DNA yield more than 4µg is required. They also found that the LAMP reaction can produce DNA yields of ≥ 10 µg compared to 0.2 µg produced by conventional PCR in 25µl reaction. On the basis of this reaction, Tomita *et al.,*(2008), developed a colorimetric method for detection of LAMP reaction by adding Calcein (fluorescence metal indicator) to the pre-reaction solution in conjunction with manganese ions.

2.1.4.2 Gel electrophoresis and visualization

In addition, to confirm that the DNA amplified in the reaction is the target DNA and not the non-specific amplification or primer dimers, the final product can be visualized on the gel to obtain the characteristic banding pattern defined by the number of target sequence copies (Tomlinson, 2008). Amplified DNA also can be detected by intercalating dyes such as, ethidium bromide (Pham *et al*., 2005), SYBR green dye (Iwamoto *et al*., 2003), Hydroxynaphthol Blue (HNB) (Goto *et al*., 2009) and Pico Green (Tsai *et al*., 2009).

2.1.5 Advantages of LAMP

The principal characteristic of the LAMP amplification is its competence to amplify nucleic acids under isothermal conditions in the range of 65°C and thus allows the use of simple and cost effective reaction equipment like water baths and heating blocks (Notomi *et al.,* 2000). This assay has a high specificity and high amplification efficiency. In terms of specificity it is so high because it can amplify a specific gene from a human genome specimen discriminating a single nucleotide difference (Parida *et*

al., 2008). High amplification efficiency is due to no time loss of thermal change because of its isothermal reaction. The reaction can be conducted under optimal temperature of the enzyme and the inhibition reaction at the later stage of amplification is less likely to occur compared to Polymerase Chain Reaction (Parida *et al.,*2008).

Visualization is easy in LAMP amplification, as the LAMP reaction progresses, turbidity increases and thus making it easier to monitor through the naked eye. In addition both amplification and detection of gene can be completed in a single step by incubating the mixture of gene sample, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (Nagamine *et al.,*2002). LAMP assay yields high results with DNA being amplified 10^9-10^{10} times in 15 to 60 minutes. LAMP assay also has the advantage of monitoring amplification by SYBR green 1 dye mediated naked eye visualization and by real time monitoring by an inexpensive turbid meter (Parida *et al*., 2008).

2.2 Diagnosis of hookworms

The mainstay in diagnosis of hookworm infection rests on microscopic visualization of eggs in stool. These techniques have the advantage of enabling the number of eggs per gram of faeces and the intensity of infection to be estimated (Goodman *et al*., 2007). Several methods are available for determining the presence and intensity of hookworm infections**.** Among the mostly used methods based on this principle are Kato-Katz, ether-based concentration and FLOTAC (Utzinger *et al*., 2008). Kato-Katz (Katz, Chaves, & Pellegrino, 1972) has been the recommended method of diagnosis by the World Health Organization (W.H.O,2008) for a long time but recently studies on comparison among other techniques have proved that it is not the most sensitive and accurate method of egg counts (Levecke *et al*., 2011). Recent reports suggest that the sensitivity of a single Kato-Katz thick smear for hookworm diagnosis is low, particularly in cases of low infection intensities (Booth, Vounatsou, N'goran, Tanner, & Utzinger, 2003). Kato-Katz technique is very time consuming, because the method requires between 1 to 2

hours before the glycerin clears the background of the stool smear on the slide for accurate visualization, this is problematic since few hours after the preparation of the slide, hookworm eggs are difficult to recognize due to over clarification by glycerin (Endris *et al.*, 2013). The small amount of stool analyzed (usually 41.7 mg) explains why the Kato-Katz technique has a low sensitivity to detect eggs whenever they are present at low frequency or appear highly clustered. Recent data also suggests that the Kato-Katz technique has drawbacks for screening stool samples from infants, as helminth infection intensities tend to be low and stool samples are more liquid than those collected from school-aged children or adults (Goodman *et al.,* 2007).

The ether-based concentration method (Allen and Ridley, 1970) is also widely used for diagnosis of helminths and intestinal protozoa and has the advantage of fixing the eggs or cysts (Cheesbrough Monica, 2005), and is important in remote settings where microscopy may not be readily available. As much as the formol ether concentration is a good technique in diagnosing intestinal worms, it is weighed down by shortcomings like cost of running the test, which involves the need for a centrifuge, constant electric supply, a well-ventilated work space, adequate water supply, a standard light microscope and reagents, thus doesn't make the technique suitable for resource-poor communities (Cheesbrough Monica, 2005).

The FLOTAC technique allows quantification of eggs and/or larvae of nematodes and trematodes as well as cysts and oocysts of intestinal protozoa in up to 1 g of faeces. Thus it is considerably more sensitive than the aforementioned diagnostic techniques (Utzinger *et al.,*2008). This method is considered more sensitive than Kato-Katz because a single FLOTAC allows a 24-fold higher amount of stool to be examined than a single Kato-Katz thick smear (Glinz *et al.,* 2010).

These microscopic methods of diagnosis cannot diagnose hookworm eggs to the species level or distinguish them from other *strongylid* nematodes that infect humans (Gasser, 2006) to overcome this limitation, larval culture can be utilized to enable eggs to develop to third stage larvae (L3s) which can be identified microscopically to the genus

level (Gasser *et al.,* 2008). This technique, however, takes about a week to perform, it is laborious, unreliable and requires skilled personel to microscopically identify and differentiate larvae (Gasser *et al.,*2008).

There are immunological methods available for the diagnosis of hookworm and other soil transmitted helminths (Bungiro & Cappello, 2005). These serological techniques are based on the detection of specific *in-vitro* antigen-antibody reactions. Among the serology-based technique is the feacal ELISA that is of high sensitivity as described by (Bungiro & Cappello, 2005) that could detect as low as 10ng/ml of excretory/secretory (ES) antigens demonstrating that the technique can be used to diagnose low intensity infection that might not be detected by conventional microscopy, allowing for more accurate estimates of hookworm prevalence. Serological techniques are most likely to predict active infection (Coulibaly *et al.,* 2013). However these methods are not able to achieve the differentiation between current and recent past hookworm infections, and usually suffer from problems of cross-reactivity between co-infecting helminth species (Gasser *et al.,* 2008). These approaches may be complicated by need to obtain blood, persistence of antibody responses following cure by chemotherapy (Geiger, Massara, Bethony, Soboslay, & Corrêa-Oliveira, 2004). They are also difficult in evaluating results especially when a patient originates from areas where the infection is endemic (Bruschi & Castagna, 2004).

In the recent past, advancement has been made in the development of DNA-based tools for the diagnosis of hookworm infection in humans (R B Gasser *et al*., 2008). Modern molecular technologies have a substantial impact in many fundamental and applied areas of parasitology. In particular, polymerase chain reaction (PCR)-coupled approaches have found broad applicability because their sensitivity permits the enzymatic amplification of gene fragments from minute quantities of nucleic acids (R B Gasser *et al*., 2008). The key to the molecular identification of parasite to species is the selection of a suitable genetic marker or locus. Since different genes evolve at different rates, the DNA region selected should display sufficient sequence variability to enable the identification of

parasite to the taxonomic level required (R B Gasser *et al*., 2008). Taxonomic studies of *strongylid* nematodes, including human hookworms have mainly used ITS-1 and ITS-2 of nuclear rDNA as genetic markers for specific identification (Chilton, 2004). These two loci are particularly suited for the development of an effective, specific and sensitive diagnostic PCR, given they are short (~250-600 base pairs) and repetitive but homogenized as a consequence of concerted evolution (Robin B Gasser, 2006).

A study conducted by Verweij *et al.*, (2001) applied a semi nested PCR to estimate the prevalence of *N. americanu*s and *Oesphagostomum bifurcum* in humans in Ghana. De Gruijter *et al*., (2005) undertook an epidemiology survey using semi nested PCR assay specific for each *N. americanus* and *A. duodenale.* Later Verweij *et al.,*(2007) established a multiplex real-time PCR assay for the detection and quantification of genomic DNA of *N. americanus, A. duodenale*, and *O. bifurcum* in the faeces from infected humans. These PCR based methods in the above-mentioned studies proved to be more sensitive than the conventional methods in the diagnosis of hookworms.

They demonstrated the utility and merits of molecular technologies as diagnostic tool for clinical purposes and epidemiology tools to estimate the prevalence and distribution of hookworms in humans and to estimate infection intensities and monitor the effectiveness of treatment programs.

Establishment of molecular assays in particular those employing genetic markers in nuclear rDNA provide a foundation for developing enhanced tools to overcome the major limitations of traditional coprodiagnostic techniques (R B Gasser *et al*., 2008).

The shortcoming with PCR based molecular tools are the expensiveness of the assay, since the equipments required are quite costly, also the aspect of time, PCR-based tools, take a long time to perform. At this stage, it appears that a practical and efficient platform for both laboratory based assay and field based assay is the loop mediated isothermal amplification, since it addresses the drawbacks observed in PCR and other diagnostic tools. Loop mediated isothermal amplification technique has been applied in the diagnosis of may parasitic diseases with significant success, so this study aims to bridge the diagnostic gap by applying LAMP in the diagnosis of hookworm, specifically *N. americanus* with great sensitivity, specificity and with reduced time.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study area was Msambweni constituency of Kwale County which is approximately 50.3 kilometres south of Mombasa town. Matuga and Lungalunga constituencies border Msambweni to the south while Kinango borders it from the north. Msambweni has a coverage of 729 km^2 and lies at coordinates of 4.4700° S, 39.4800°E (Mtange, 2014). Hookworm and other soil-transmitted helminths (STHs) are endemic in this area particularly among school going children and pre-school children. Kwale has previously been shown to be highly endemic for STH among school going children (Njenga *et al*., 2011).

3.2 Study Population

Children between the ages of 6 and 15 years attending madrasas (informal Muslim schooling system) and whose parents/guardians had given informed consent for them to take part in the study were sampled. A total of seven (7) madrassas namely; Marigiza, Magaoni, Bodo, Najaha, Mchinjirini, Shirazi and Kingwede, were selected for stool sample collection based on their prevalence of hookworm and other soil transmitted helminths (STHs). Children who were between the ages of 13 years to 15 years old were also asked to give assent if they accepted to take part in the study.

3.2.1 Inclusion Criteria

Hookworm positive school going children infected with hookworm aged between 13 to 15 years and whose parents/ guardians had given written consent for them to participate in the study and who assented to participating in the study were recruited.

3.2.2 Exclusion Criteria

Children who were not infected with hookworms and those whose parents/ guardians declined to issue a written consent for their children to take part in the study were excluded.

3.3 Parasite collection and diagnosis

Children within the selected madrasas and who met the inclusion criteria were issued with polypots where they put stool sample. These madrasas were chosen based on hospital records that showed high infection intensities. The samples were then transported to the field laboratory based at the Msambweni District Hospital for diagnosis. The samples were processed and checked for the presence of hookworms using the Kato-Katz technique (Katz *et al*., 1972).

The Kato-Katz slides for microscopy were prepared in duplicates, I read one slide and the other by a trained technician based at Msambweni District hospital. Also, 5% of the slides were read by a third technician for quality control purposes. In carrying out the Kato-Katz, the stool was gently mixed and pressed through a stainless-steel sieve with 200µm mesh to remove fibres and other debris. A standard plastic template that takes a total of 41.7mg was placed on a labelled microscope glass slide and stool carefully put to fill the template hole. The template was carefully removed and a cellophane strip that had previously been soaked in malachite green was placed over the feacal sample. The slide was inverted onto a blotting paper and pressure applied gently and evenly to spread the feacal sample to about half an inch in diameter. This could sit for 20 minutes then observed under the microscope at a magnification of X10 for the detection of parasite ova. The eggs observed were counted for each slide to determine infection intensity. The numbers of eggs counted were multiplied by 24 to get the number of eggs per gram (epg) of faeces. The observations made from Kato-Katz were recorded in single ruled exercise books and backed up in excel sheets in a password protected laptop. The stool

samples were stored overnight in a -80°C chest freezer. The samples then transported in cool boxes and covered with icepacks, back to the *Schistosomiasis* laboratory based at the Kenya Medical Research Institute (KEMRI) in Nairobi for Molecular analysis.

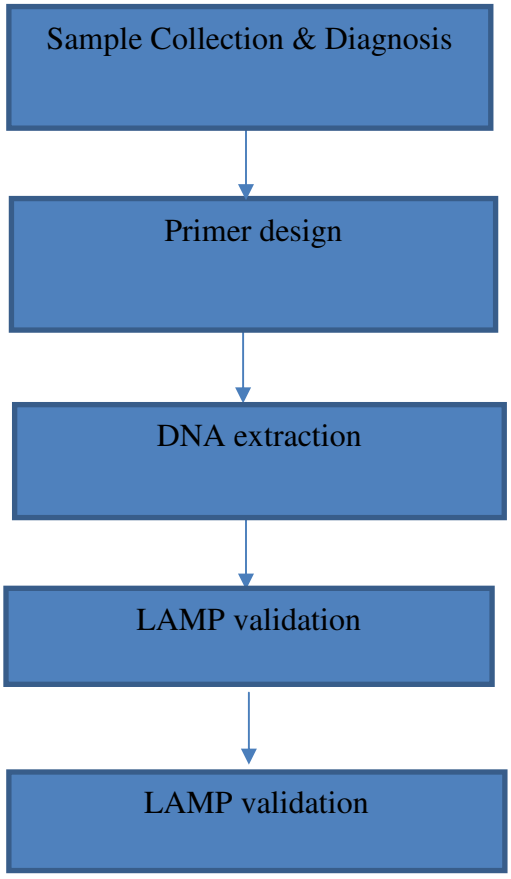


Figure 3.1: Flow diagram showing flow of work done

3.4 Molecular analysis

3.4.1 Primer design

A total of 19 sequences of Internal transcribed spacer 2, accession numbers; KC896820.1-KC 896825.1, Y11734, AF217891, HQ452515-HQ452517, HQ452537-HQ452543 and AJ001599, were selected from the National Centre for Biotechnology Information (NCBI). These sequences were subjected to multiple sequence alignment (MSA) using a program called CLC main workbench version 7.0 and a consensus sequence was developed (Table 3.1). LAMP. Primers were designed using Primer Explorer version 4.0 software (http://primerexplorer.jp/elamp 4.0.0). The internal transcribed spacer (ITS-2) gene of the ribosomal DNA (rDNA) was chosen for amplification because of its low mutation rates and substantial differences between species (R B Gasser et al., 2008).

Table 3.1: Consensus sequence of ITS-2.

The consensus sequence of ITS-2 is used for development of LAMP primers

Sequence name	**Sequence length (BP)**	**Consensus sequence**
ITS-2	374 base pairs	**1** CACGTCTGGTTCAGGGTTGTTAACGATAATACTACAGTGT AGCTTGTGGA CAGTACTCTC **61** ACCGAGTATTGTTGAACACTGTTTGTCGAACGGTACTTGC TCTGTACTAC GCATTGTATG **121** TGTTGTGTTCAGCAATTCCCGTTTAAGTGAAGAACACAGT GCAACATGTG CACGCTGTTA **181** TTCACTACGTTAGTTAGCTAGTTTACTAACGTATGATAGCGGTGCATAC T GTATGACATG **241** AACATATCGTTGTTCACTGTTTAATCGCTCTCGCGACTTA TGAGCGTGGTTGAACGGAGA **301** CAATGTGAAGGACAACGATGTTCGCCATGTGGATGTGTCA TTTGCAATGC AACCTGGCTC **361** AGGCGGATTA CCCG

This consensus sequence was inputted into the LAMP primer design software, primer explorer version 4.0 (Notomi *et al*., 2000). Five sets of primers were generated by the software (Table 3.2) and synthesized.

Table 3.2: Primers generated by the LAMP software.

The five sets of primers generated the software.

Primer Name	Primer length	Primer sequence
Set 1	22 22 41 38	**F3**: AGTATTGTTGAACACTGTTTGT **B3**: AACAACGATATGTTCATGTCAT **FIP**: CACTTAAACGGGAATTGCTGAAC-CGAACGGTACTTGCTCTG **BIP**: GCAACATGTGCACGCTGTTA-ACAGTATGCACCGCTATC
Set 2	18 18 42 43	**F3**: TCGAACGGTACTTGCTCT **B3:** TTGTCTCCGTTCAACCAC **FIP**: CATGTTGCACTGTGTTCTTCAC- CTACGCATTGTATGTGTTGT **BIP**:GTTTACTAACGTATGATAGCGGTGC-CTCATAAGTCGCGAGAGC
Set 3	19 24 48 43	**F3**: TGGTTCAGGGTTGTTAACG **B3**: GTTAGTAAACTAGCTAACTAACGT **FIP**:TTCGACAAACAGTGTTCAACAATAC-ATAATACTACAGTGTAGCTTGTG **BIP**: CTGTACTACGCATTGTATGTGTTGT-AGTGAATAACAGCGTGCA
Set 4	19 18 43 45	**F3:**TGCTCTGTACTACGCATTG **B3:**AACCACGCTCATAAGTCG **FIP:**TGAATAACAGCGTGCACATGTTG-TATGTGTTGTGTTCAGCAAT **BIP:**AGTTTACTAACGTATGATAGCGGTG-CGAGAGCGATTAAACAGTGA
Set 5	19	**F3:**TGTTGTGTTCAGCAATTCC

	18	**B3:**GCGAACATCGTTGTCCTT
	46	**FIP:**CACCGCTATCATACGTTAGTAAACT-CGTTTAAGTGAAGAACACAGT
	44	**BIP**:TGACATGAACATATCGTTGTTCACT-ATTGTCTCCGTTCAACCAC

The primers generated were tested for their suitability and reliability and of the five (5) sets of primers, primers set 1 gave the best results in terms of reliability and reproducibility and was used for further analysis in LAMP technique development.

3.4.2 DNA extraction

DNA was obtained from feacal samples that were specifically positive for hookworm infection by Kato-Katz. Extraction was done using QIAMP DNA stool kit. The extraction process was carried out as per the manufacturer's instruction. Briefly, 200 grams of stool was weighed and placed in a 2ml Eppendorf tube. To the weighed stool sample, 1ml of EX buffer was added and vortexed for 1 minute. The homogenized solution was heated for 5 minutes at 95°C, after which it was vortexed for 15 seconds. This was followed by centrifugation for 1 minute at 14,000 rpm to allow the debris to settle at the bottom of the tube. In a new 1.5 ml, sterile tube, 15µl of proteinase K was added followed by addition of 200µl of the supernatant from the centrifuged stool solution and a further addition of 200µl of buffer AL. This was then vortexed for 15 seconds to mix all the cell content. This solution was then incubated for 10 minutes at 70°C after which 200 µl of absolute ethanol was added and the resultant mix vortexed. 600µl of the resulting lysate solution was pippeted into a spin column and centrifuged for 1 minute at 14,000 rpm. The filtrate was then discarded. To the same spin column 500µl of buffer1 (AW1) was added and centrifuged for 1 minute and the filtrate discarded followed by addition of 500µl of buffer 2 (AW2). This was then centrifuged for 3 minutes and the filtrate discarded. The collection tube on the spin column was then

replaced with a new one and a dry spin was done for 3 minutes at 14,000 rpm to ensure that any of the buffers used were completely removed. After the dry spin, 200µl of DNA elution buffer was added then incubated at room temperature for 1 minute followed by centrifugation for 1 minute at 14,000 rpm to elute the DNA. The DNA eluted was quantified using a NanoDrop 2000 UV-Vis spectrophotometer and aliquoted into sterile PCR tubes. The resultant DNA was stored at -20^{o}C until used in subsequent molecular analysis.

3.4.3 LAMP Optimization

The LAMP reaction was carried out using a gene Amp 9700 thermocycler. A reaction mix was first prepared in a final reaction volume of 18µl. The components of this reaction mix were as follows: 40 pmols of each inner primer FIP and BIP and 10 pmols of each outer primer F3 and B3, 10X *Bst* buffer, 2X reaction buffer and 1Unit of *Bst* polymerase enzyme. The LAMP optimization was done by varying the temperature and time. The concentration ratio of the inner and outer primers was done as per Notomi *et al*. (2000). The temperature variation range was between 59°C to 65°C. This was done till the temperature that gave the best results, in terms of well amplified products, was achieved. The same was done in determining the least time the assay would take to complete while achieving the best desired results. The time range was varied from 45 minutes to 120 minutes. After the optimization process, the following best LAMP conditions were adopted: 95°C for 5minutes, followed by 63°C for 60 minutes for the amplification to happen, and a final incubation at 80°C for 10 minutes to stop the reaction.

3.4.4 Amplicon visualization

The amplicons were separated using electrophoresis on a 2% agarose gel. To prepare the agarose gel, 1.0g of agarose powder was weighed and dissolved in 50ml of 0.5X TAE buffer. The mixture was swirled gently and heated in a microwave for 2 minutes to melt

the agarose. The mixture could cool after which 1µl of ethidium bromide, an intercalating nucleic acid stain, was added. The warm agarose solution was poured into a gel casting mold, and placed well combs and this could solidify at room temperature. The solidified gel was placed into an electrophoresis tank containing 0.5X TAE buffer. The amplified samples were then loaded into the wells on the gel. Prior to loading, 5µl of the sample was mixed with 1µl of 6X loading dye and then loaded into the wells on the gel. To the first well, a ready to use 100bp molecular ladder was loaded to help determine the sizes of the fragments of amplified DNA. The samples could run for 40 minutes at 95mV followed by visualization under a bench-top UV transilluminator.

The second method that was used to view the amplified products was SYBR green 1 a nucleic acid stain obtained from Invitrogen. SYBR green 1 changes colour from orange to green in the presence of positively amplified DNA and remains orange if the sample is negative. The SYBR Green stain was diluted with PCR water to make the working solution in the ratio 1:10. 1µL of this was added to each of the DNA sample. A negative control having distilled water was included for all the reactions.

3.4.5 LAMP validation

For validation of the LAMP technique, fresh stool samples were collected from school children from Msambweni area, an endemic area for hookworm infection. The optimized conditions described in section 3.4.3 above were used to analyse stool samples that had tested either positive or negation by Kato-Katz. A total of 106 stool samples were used in validating the LAMP technique. The samples were determined as positive or negative using the Kato-Katz technique as described in section 3.3 and the data recorded in a laboratory book and backed up in a password protected laptop. These samples were then subjected to molecular analysis using the LAMP technique under the following conditions; 95°C for 5minutes, followed by 63°C for 60 minutes for the amplification to happen, and a final incubation at 80°C for 10 minutes to stop the reaction. The amplicons were separated on a 2% gel agarose stained with ethidium

bromide and using SYBR green 1 as described in section 3.4.3. The products were recorded by way of photography.

3.4.5.1 Specificity of the LAMP technique

To verify the specificity of the LAMP assay for the detection of *N. americanus* DNA, the LAMP primers specific for hookworms were used to amplify DNA from related helminths specifically *S. mansoni, A. lumbricoides* and *T. trichiura*. The *S. mansoni* DNA was obtained from the *Schistosomiasis* laboratory based at the Kenya Medical Research Institute while samples of *A. lumbricoides* and *T. trichiura* were obtained from feacal samples collected from school children from the same study population. These samples were amplified under the same conditions as described in section 3.4.3. The amplicons were viewed on a 2% agarose gel and SYBR green 1 stain and documented by photography. Percentage specificity was calculated using the formula below: -

$$\text{Specificity} = \frac{\text{True negatives}}{\text{True negatives + false positives}} \times 100$$

3.4.5.2 Sensitivity of the LAMP technique

To determine the sensitivity of the LAMP technique, DNA was extracted from a stool sample that was positive for hookworm by Kato-Katz technique as described in section 3.4.2. The DNA extracted was quantified spectrophotometrically using a Nano Drop 2000 cc after which it was serially diluted 10 folds using PCR water from an initial concentration of 40ng/µl. The resultant dilutions were analyzed using the LAMP technique under the same conditions as described in section 3.4.3. The samples after amplification were observed on a 2% agarose gel and viewed under a bench top UV trans illuminator and a photo of the gel was taken and saved. Percentage sensitivity was calculated using the formula below: -

$$\text{Sensitivity} = \frac{\text{True positives}}{\text{True positives + false negatives}} \times 100$$

3.4.6 Data collection and analysis

Sensitivities and specificities were estimated using the Kato-Katz method as the reference test. The sensitivity was defined as the percentage of samples with a true positive result, while the specificity was defined as the percentage of samples with a true negative result. Kappa statistic was used to compare the level of agreement between the two diagnostic assays that is Kato-Katz and LAMP. The following kappa statistic formulae was used to achieve the level of agreement

$$\text{Agreement level} = \frac{\text{(\% Observed Agreement.) – (\% agreement Expected by chance)}}{\text{100\% - (\% agreement Expected by chance)}}$$

3.4.7 Ethical Consideration

Approvals for the study were obtained from the Scientific and Ethical review committee (SERU) at the Kenya Medical Research Institute (referenced SSC No. 2683, appendix 3). Participation in the study was voluntary and informed consent was sort from the parents of all participating pupils. Assents were also obtained from the children. All the children found to be infected with hookworms or any of the soil-transmitted helminths received a 400mg albendazole, in a single dose, as treatment for soil-transmitted helminths, under the supervision of a clinician. Feacal sample collection for STH infection diagnosis is a routine procedure for parasitological investigations and does not pose any risks or cause any harm to participating children.

CHAPTER FOUR

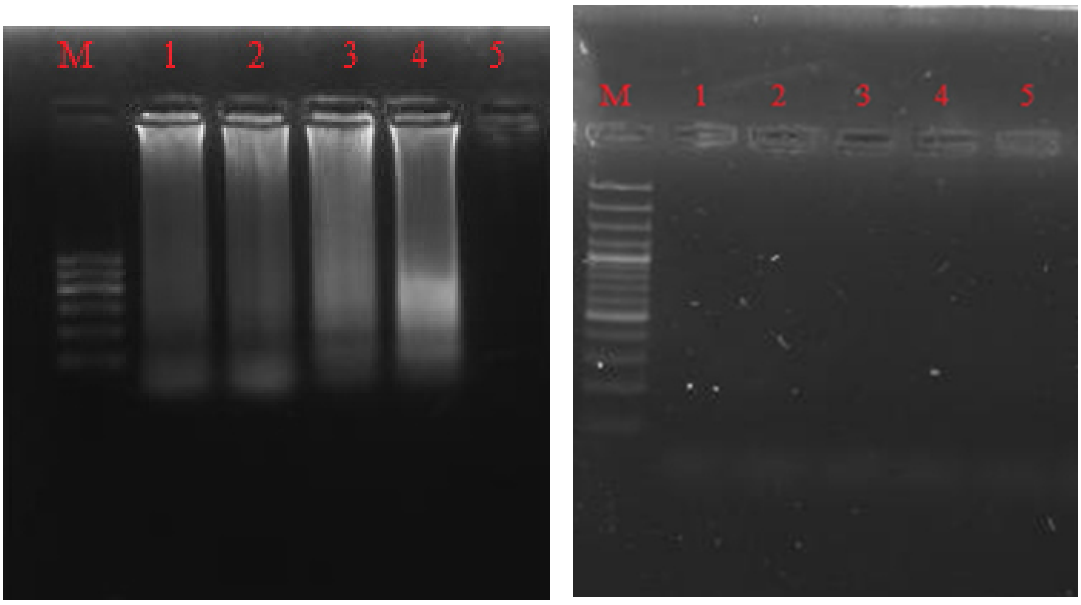
RESULTS

4.1 Primer Selection for the LAMP Reaction

In this study, a total of 5 sets of primers (Table 3.2) were designed based on Internal transcribed spacer of rDNA and tested for LAMP amplification. Among these primers, the set that had the highest amplification rates and the shortest amplification time was selected for analysis. Primer set 1 (Table 3.1) gave the best results in terms of high sensitivity and specificity. It also displayed the shortest amplification time and the highest product yield hence this primer set was used in all the subsequent analysis.

4.1.1 Temperature Selection

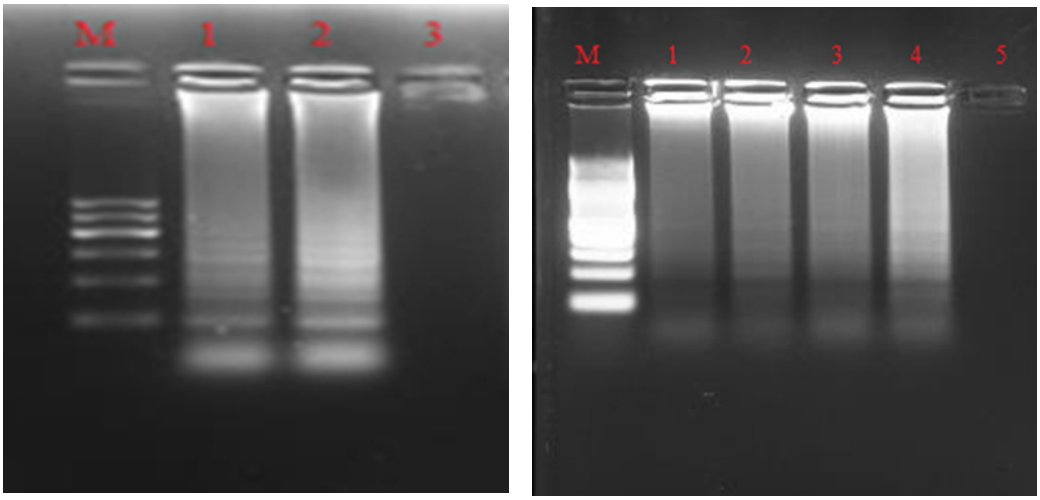
Based on the GC content of the primers and their length, temperatures ranges between 59°C to 65°C were tested. At 59°C and 60°C the amplicons appeared as smears when the samples were running on a 2% agarose gel as shown in figure 4.1(a). At a temperature of 61°C primer dimers were observed on a 2% agarose gel after amplification figure 4.1(b). At 62°C, bands were observed but they were not clear enough to distinguish using a 100-base pair molecular marker as in figure 4.2(a). At 64°C and 65°C amplicons observed showed smears and non-specific amplifications as in figure 4.2(b).



(a) (b)

Figure 4.1: Sample amplification at 59^0C, 60°C and 61^0C.

(a) Shows amplification done at 59°C for sample 1 and 2 while 3 and 4 were done at 60°C. Number 5 is the negative control. (b) Shows amplification at 61ºC. Sample 1,2,3,4 are hookworm samples while 5 the negative control.



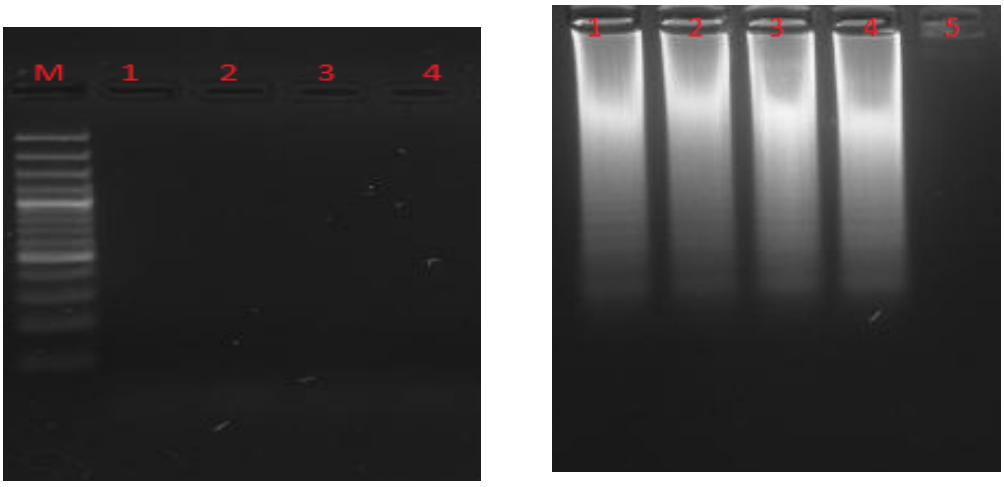
(a) (b)

Figure 4.2: Sample amplification at 62^0C, 64^0C and 65^0C.

(a) Shows amplification done at 62ºC for sample 1 and 2 while 3 is the negative control. (b) Shows amplification at 64ºC for sample 1 and 2 while 3 and 4 were carried out at 65ºC, 5 the negative control.

4.1.2 Time Selection

The optimum duration of the assay was also tested to determine the best duration for the assay. Using samples that had previously been determined to be positive, the time ranges tested were; 45, 60, 90 and 120 minutes. At 45 minutes, there were no bands on a 2% agarose gel (Figure 4.3.) At 90 and 120 minutes the amplicons appeared as smears and not the characteristic ladder-like bands. The optimum time gave high product yield with no nonspecific amplification was 60 minutes as the bands observed were clear and had the ladder-like characteristic of LAMP reactions (Figure 4.4)



(a) (b)

Figure 4.3: Sample amplification at 45, 90 and 120 minutes.

(a) Shows amplification carried out at 45 minutes at 63°C. Sample 1, 2 & 3 were hookworm positive, while sample 4 was the negative control. Photo (b) shows amplification done for 90 minutes for sample 1 and 2 and 120 minutes for 3 and 4 at 63ºC, sample 5 was the negative control.

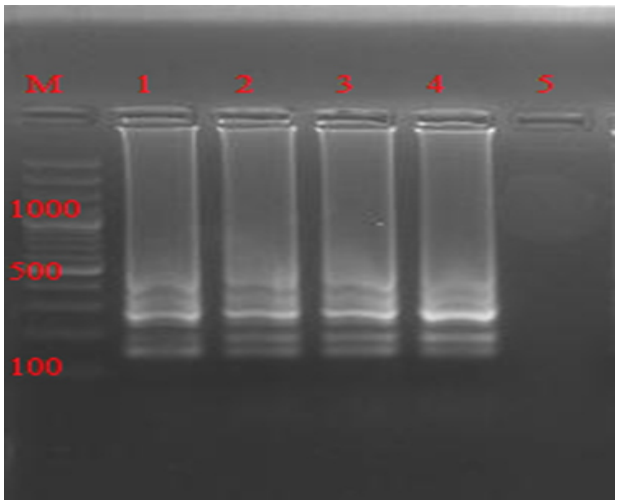


Figure 4.1: Amplification at 63^0C for 1 hour.

Lane 1- 4 shows human feacal samples containing *N. americanus* DNA. Lane 5 is the negative control.

The ladder- like appearance of the bands is due to the formation of various sized structures consisting of alternately inverted repeats of the target sequence (ITS-2) ranging from ~150 to ~600 base pairs.

4.1.3 Amplicon visualization using SYBR green

Other than gel electrophoresis SYBR green nucleic acid stain has been shown to be able to detect positive LAMP amplicons by colour change from orange for negative samples to green for positive samples. In this study, it was possible to visualize using SYBR green (Figure 4.5) due to the production of magnesium pyrophosphate with is a by-product of LAMP reactions

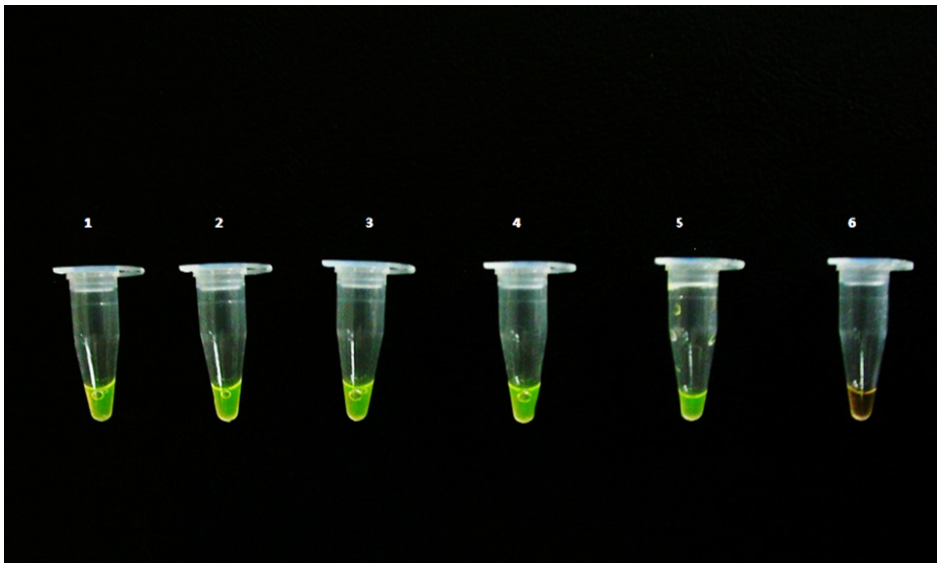


Figure 4.2: Amplicon visualization using SYBR green.

Amplicons visualized using SYBR green nucleic acid stain following LAMP amplification.

4.2 Specificity of the LAMP Reaction

The specificity of the reaction was determined by subjecting DNA from *N. americanus* and other related helminths, namely; *A. lumbricoides, S. mansoni and T. trichiura* under the optimum LAMP conditions for hookworm as described in section 4.1. The amplicons were viewed on a 2% agarose gel. Only DNA samples from *N. americanus* were amplified but not the samples from the related helminths implying that there was no cross reactivity of the assay with the other helminth parasites (Figure 4.6)

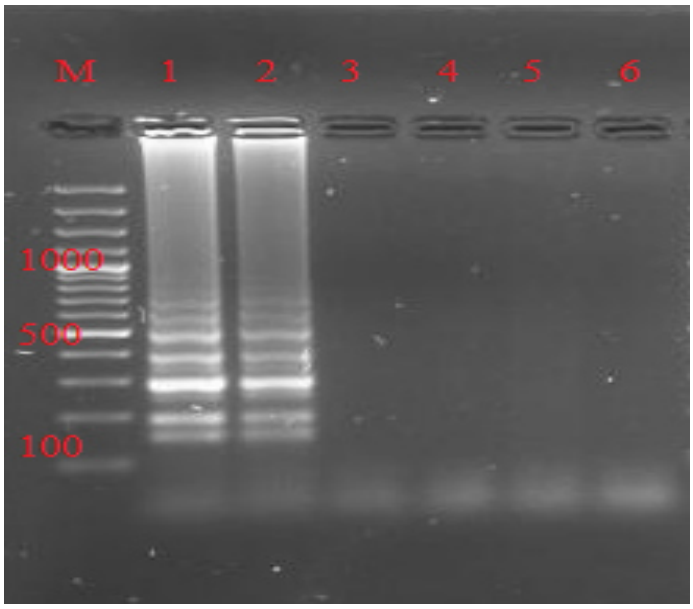


Figure 4.3: Specificity of the LAMP Reaction.

Specificity of the assay following LAMP amplification of related helminth parasite. Lane 1 and 2 shows *N. americanus* DNA, Lane 3 shows *S. mansoni* DNA, Lane 4 shows *A. lumbricoides* DNA, Lane 5 shows *T. trichiura* DNA and Lane 6 shows Negative control.

4.3 Sensitivity of the LAMP Reaction

Tenfold serial dilutions of *N. americanus* DNA was used to test the sensitivity of the LAMP assay. The detection limit of the assay was as low as 0.4fg when the samples were run on a 2% agarose gel (Figure 4.7).

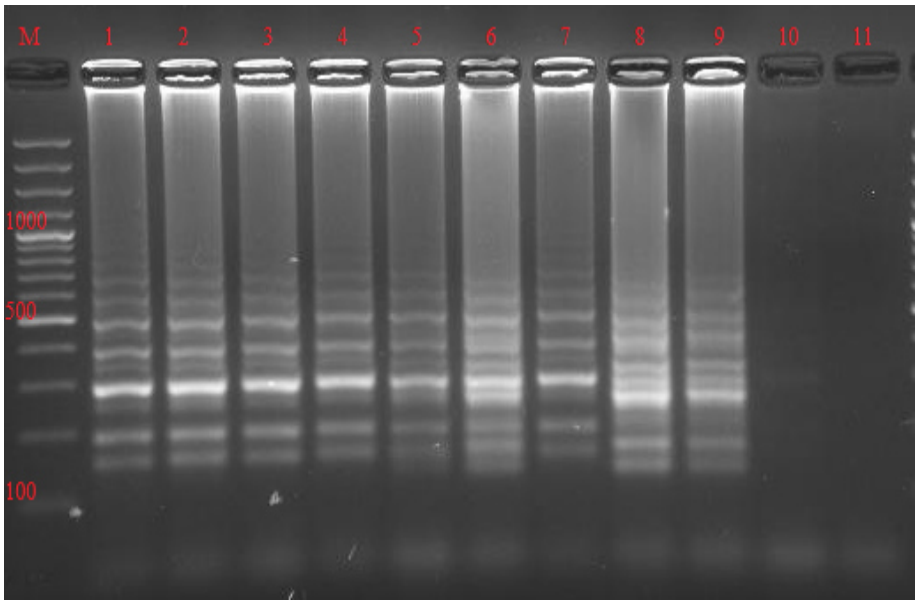


Figure 4.4: Sensitivity of the LAMP Reaction.

Shows products from ten-fold serially diluted concentrations of *Necator americanus* DNA. Lane 1 a concentration of 40 ng of DNA was used, Lane 9 is 0.4 FG and Lane 11 is a negative control.

4.4 Comparison of Loop mediated isothermal amplification (LAMP) and Kato-Katz

To validate this technique, a total of 106 stool samples collected from Msambweni were analysed by comparing the LAMP assay with the Kato-Katz. These samples were first diagnosed for hookworm infection by Kato –Katz and later the same samples were subjected to LAMP assay. Out of the 106 samples, 86 samples were found to be positive by Kato-Katz technique, while 83 of the samples were found to be positive by LAMP. 20 samples were found to be negative for hookworms by both techniques Kato-Katz and LAMP (Table 4.1)

Table 4.1: Levels of agreement between LAMP method and Kato-Katz method.

A contingency table showing the level of agreement between LAMP method of diagnosis and Kato-Katz which is the gold standard used in the diagnosis of hookworms.

Kato-Katz

		+	**-**	**TOTALS**
LAMP	**+**	83 (**True positives**)	0 (**False negatives**)	83
	-	3 (**False negatives**)	20 (**True negatives**)	23
	TOTALS	86	20	**106** **(Total)**

In terms of sensitivity LAMP showed a sensitivity of 97% while in terms of specificity LAMP was 100% specific. Also, the agreement between the two techniques was determined by calculating the Kappa coefficient as described by Viera and Garrett (2005).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

The gold standard of hookworm diagnosis has been mainly based on the detection of the ova in human feacal samples. Despite the wide application of this technique it is too laborious, lacks sensitivity to detect low level infections, and lacks specificity, since it cannot differentiate between ova of *Ancylostoma duodenale* and *Necator americanus.* The technique also requires well trained and experienced laboratory technicians (Tarafder *et al*., 2010b). Flotation method of diagnosis, that is formal ether concentration, is not reliable in hookworm diagnosis because the preservation medium, sodium acetate-acetic acid formalin decreases feacal egg count (Glinz *et al.,* 2010). Serological methods are available but they are unreliable since they cannot differentiate between past and present infection and can be misleading since some patients suffer from problems of cross-reactivity (Coulibaly *et al*., 2013a). Molecular based techniques such as the Polymerase chain based techniques have been developed (Wang, Pan, & Cui, 2012a) they, however, require expensive equipments, are time consuming and are not applicable in resource-poor settings. An accurate, specific and sensitive diagnosis system for hookworm is critical for accurate detection of hookworm following mass drug administration when infection intensities are lowered following chemotherapy. Currently the World Health Organization's recommended strategy in the control of *schistosomiasis* and Soil Transmitted Helminths is by mass drug administration (MDA) (W.H.O, 2008). One notable result of MDA is the lowering of infection intensity both in individuals and populations under treatment. This requires diagnostic methods that are sensitive enough to detect infections at very low intensities.

In this study, we have developed a sensitive and highly specific LAMP assay that can detect hookworm infection specifically, *N. americanus* DNA in feacal human samples.

This LAMP technique is based on the internal transcribed spacer-2 (ITS-2) of the ribosomal DNA. This region was chosen because of its low mutation rates within species and substantial differences between species (R B Gasser *et al*., 2008). Key to molecular identification of parasites to species level is selection of a suitable genetic marker or locus. Different genes evolve at different rates, therefore the DNA region selected should display sufficient sequence variability to enable the identification of parasites to the taxonomical level needed. For specific identification, the locus should display no or very minor variation within a species but differ at higher levels in sequence among species (Gasser, 2006).

In taxonomic studies of human hookworms have used ITS-1 and ITS-2 of nuclear rDNA as a genetic marker for specific identification, since they have demonstrated to have been highly conserved and can distinguish between the two human hookworm species, that *Necator americanus* and *Ancylostoma duodenale* (Hawdon, 1996). In this study ITS-2 preferred to ITS-1 as a genetic marker suited for the development of an effective, specific and sensitive diagnostic LAMP assay. Primers were designed from both loci but ITS-2 primers were more robust. ITS -2 is short and repetitive locus ranging in size of about 250- 600 base pairs as shown in the gel photos. The primers designed were highly specific and sensitive.

The assay was optimized to amplify DNA in 1 hour but this time could be significantly reduced if loop primers were involved. According to (Nagamine *et al*., 2002), the use of two additional loop primers, that is, Loop inner primer and loop outer primer will reduce the amplification time taken. This was done using a thermocycler, since the study was a proof of concept that the assay can be utilized in clinical diagnosis of *N. americanus* from stool, but under isothermal conditions the assay can be performed using a simple incubator, such as a water bath or a heat block thus making it cost effective.

Hybridization of the primers to the target DNA in the initial stages is important for the efficiency of the assay. The sequence and sizes of the primer were chosen so that their

melting points fell within the ranges of 59℃ to 65℃ as described by (Notomi *et al*., 2000). At 63℃ the assay gave the results when viewed on agarose gel. The bands formed a ladder-like pattern, this is because of the formation of various sized structures consisting of alternately inverted repeats of the target sequence.

To visualized amplified products, SYBR Green dye and agarose gel were used. Using SYBR green dye, the positive products change colour from orange to green. While in the agarose gel ladder like bands were observed as described above. Notomi *et al.,* 2000 demonstrated that visualization with the naked eye is possible due to the formation of magnesium pyrophosphate, which is a by-product of the reaction. This removes the need for agarose gel electrophoresis and SYBR green dye thus adapting the technique for potential use at a field set up.

The LAMP assay optimized in this study was then compared to Kato-Katz which is the gold standard of hookworm diagnosis. A total of 106 stool samples were used to evaluate the technique. These samples were diagnosed using both methods, LAMP and Kato-Katz, and out of the 106 samples, 83 were deemed positive by LAMP but Kato-Katz had three more positives samples. The difference was attributed to human error, something that the gold standard is prone to. Both techniques deemed 20 stool samples as negative. From the above data, a contingency table was created (Table 4.0). The contingency table was used to calculate both sensitivity and specificity of the LAMP technique. The LAMP technique showed 97% sensitivity and 100% specificity. The table also aided in the calculating the level of agreement between the two diagnostic methods, using the formula described by Viera and Garrett, (2005), from this the kappa coefficient was found to be 0.9 which showed excellent agreement between the two.

The sensitivity and the specificity were of the LAMP assay were also determined using molecular methods. The assay demonstrated a detection limit of 0.4 femtograms of *N. americanus* DNA that was serially diluted 10 folds, starting with an initial DNA concentration of 40ng. Specificity was determined by amplifying DNA from *A.*

lumbricoides, S. mansoni and *T. trichiura* using the hookworm LAMP primers and *showed* no cross reactivity. This soil transmitted helminths were selected because they commonly appeared in the sample area and in some study participants had co-infections were also observed, upon carrying out Kato-Katz.

LAMP has been used in diagnosis of other parasitic diseases. Nkouawa *et al.,* 2010 developed and evaluated a LAMP assay for the differential diagnosis of infection with *Taenia* species. The assay was based on L-like cysteine peptidase (clp) and cytochrome c oxidase subunit 1 genes (cox1). LAMP with primer set to the clp genes could differentiate *T. solium* from *T. saginata* and *T. asiatica.* When compared to PCR, both methods showed similar sensitivity but LAMP was faster as results were obtained within an hour, and more specific as it requires four primers that recognize six different sequence on the target sequence.

Thekisoe *et al.* (2007) developed a LAMP technique for the diagnosis of both animal and human trypanosomiasis based on the 5.8SrRNA-ITS 2 gene for *T. brucei gambiense*, 18SrRNA for both *T. congolense* and *T. cruzi* and VSG RoTat 1.2 for *T. ovansi*. The technique could detect down to 1fg trypanosomal DNA. The assay was done at 63^0C for 1 hour and then at 80°C for 2 minutes to terminate the reaction in a heat block. The assay demonstrated simplicity in amplification and in visualization of amplicon by naked eye as white turbidity indicating positive amplification.

LAMP technique has also been applied in early detection of *S. mansoni* in stool samples (Fernández-Soto *et al.,* 2014). A murine model was used to test the new LAMP, because it allowed for collection of well-defined stool samples that would have been difficult to obtain from human patients. The assay was compared to Kato-Katz and ELISA. ELISA method could detect IgG antibodies in the infected mice 4weeks post infection, LAMP could detect *S. mansoni* DNA in all infected mice samples1 week post infection, demonstrating the technique's sensitivity. The assay is rapid as it was conducted at 63^0C

for 1 hour. SYBR green dye was used as the visualization method, demonstrating that LAMP can be prudentially used under field conditions in endemic areas.

This technique has also been evaluated in the diagnosis of malaria from blood samples in returned travellers. The assay was compared with nested PCR and showed that the malaria LAMP kit performed with similar diagnostic accuracy to nested PCR, but with greatly reduced time of 40 minutes in 65°C incubation (Polley *et al.,* 2013). Lau *et al.*, 2010 developed a LAMP test for clinical diagnosis of toxoplasmosis, based on SAG1, SAG2, and B1 genes. The LAMP assay developed was evaluated by comparison with conventional nested PCR. The results showed that the LAMP assay was highly sensitive and had a detection limit of 0.1 tachyzoite and no cross-reactivity with the DNA of other parasites was observed.

LAMP has also been developed for the diagnostic potential of *Babesia gibsoni,* based on the 18SrRNA. It was able to detect an estimated 0.005% parasitaemia with *B. gibsoni* specific primers (Ikadai *et al.,* 2004).

These studies have demonstrated that loop mediated isothermal amplification technique is highly sensitive, specific and takes a relatively short time to carry out. It has shown that it can be applied in clinical diagnosis of various parasitic diseases and as an epidemiological unit.

LAMP test has shown great potential for use in resource limited areas because it does not require costly equipment like thermocyclers and in areas of low level infection intensities because of its high sensitivity. The specificity and sensitivity of this assay makes it easily applicable for a clinical practice at the point of care and for epidemiological survey in areas of disease endemicity. LAMP has the characteristics that make it ideal for field work and has already been used in rural laboratories in developing areas for the diagnosis of tuberculosis (Geojith, Dhanasekaran, Chandran, &

Kenneth, 2011); with further advancement this technique can be made applicable for field set up.

5.2 Conclusion

- The ITS-2 region was successfully used to design primers and this enabled specific detection of *Necator americanus* DNA.
- The LAMP assay developed was more sensitive and specific compared to Kato-Katz technique

5.3 Recommendations

Although this study has demonstrated the potential of the use of loop mediated isothermal amplification as a diagnostic technique under laboratory setting, there is still room for improvement. I recommend that more studies should be done to include loop primers to reduce the time taken for amplification

The LAMP should also be compared to other molecular and parasitic techniques that have been used in hookworm diagnosis, for example, PCR and recently developed mini-FLOTAC kit.

Since it has shown potential, studies should be carried out to test its effectiveness in the field setting using a water bath. I recommended that further improvement can done to make it amenable for use under field conditions. Some of the improvement that can be done is to develop lyophilized reagents that will not require refrigeration. This will eliminate the need for a refrigerator, one equipment that cannot possibly be found in most field set ups where the disease is endemic.

Finally, the technique could be packaged into a portable kit. This could greatly improve mobility that could make it possible to use it at the point of care or as a door –to-door diagnostic kit.

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APPENDICES

Appendix 1: Informed Consent Form Parents/Guardian.

I, Mr./Mrs./Miss _____, being an adult aged 18 years and over, and being the parent/guardian of:

Master/Miss (Child's Name) _____ Aged_ _____,who attends_____School, do hereby give permission to Prof/Dr./Mr./Mrs./Miss _____ _____for my child to take part in the new study known as **"Development and Evaluation of a LAMP Technology-Based Test for Simultaneous Diagnosis of Schistosomiasis and Soil-Transmitted Helminthiases"** which has been explained to me in _____, a language I speak fluently and understand clearly, and now, I know what the study is all about, the tests to be done on my child, the benefits my child will receive for taking part in the study, the medications he/she will be given, if found to be sick with bilharzia or other intestinal illnesses caused by parasites, the side effects he/she could suffer from the medication, which I have been told, are mild, temporary, and should not cause any harm to my child. I was given an opportunity to ask questions and to seek clarifications of the issues I had not understood clearly about the study, and I am satisfied with the answers and the explanations I was given. I have also, been told that if I have additional questions or concerns about the study later, I can contact the researcher in charge of the study, and if I have questions or concerns about my child's rights as a participant in this study, I can contact: The Secretary, KEMRI's Ethics Review Committee, Kenya Medical Research Institute (KEMRI), P.O. Box 54840-00200, Nairobi, Phone: 020-2722541, 0722-205901, 0733-400003; e-mail: erc@kemri.org

I accept my child to take part in this study, and agree that he/she can give stool samples for the tests needed in this study. I have been told that my child can leave the

study any time he/she decides to do so, and I have been assured that he/she will not suffer any penalty or loss of benefits that he/she should get through this study. All these things have been explained to me and my child in _____, a language we speak fluently, and understand clearly. I agree to allow the researchers to remove the eggs of the bilharzia parasites from the stool samples my child will give, and they can take these eggs or the resulting adult worms, abroad for further investigations and research.

_____

Signature (or Thumb Print) of Parent/Guardian

_____

Date

_____

Name of the Person Obtaining Consent and Signature

_____

Name and Signature (or Thumb Print) of Witness

Appendix 2: Consent form for children aged 13 -15 years.

You are being asked to take part in a study called "**Development and Evaluation of a LAMP Technology-Based Test for Simultaneous Diagnosis of Schistosomiasis and Soil-Transmitted Helminthiases"** being carried out by researchers from the Kenya Medical Research Institute (KEMRI). The purpose of this study is to develop a diagnostic tool capable of simultaneously detecting bilharzia and the most common soil-transmitted nematode parasites for use in the sub-Saharan Africa, to support disease prevention and control efforts. Bilharzia is transmitted through water snails, and makes millions of people all over the world, especially children, sick.

If you agree to take part in this study, we will ask you to give stool samples so that we can check to see if you have eggs of bilharzia and soil transmitted helminth worms are in your body, and, remove eggs of the bilharzia and soil transmitted helminth worms for our experiments. If you are found to have bilharzia or other intestinal parasites, you will be given medication by the doctor to get rid of the bilharzia worms, or the other intestinal parasites present in your body, free of charge. You do not have to give a stool sample for this study, if you don't want to, but there will be no harm if you gave a sample. By giving stool, we can get to check if you have bilharzia or other intestinal parasites. Also, if you give a sample, we will be able to isolate the bilharzia eggs we need for our research. Giving stool samples will not harm you in any way. Do you agree to take part in this study and give stool samples? If you **agree to take part in this study and give stool samples, please** put a tick (√) next to the answer **"YES"**, in the space given below, and sign your name in the space provided:

YES_____I agree to take part in this study and provide stool samples.

_____ _____

Name of the Child Signature or Thumb Print

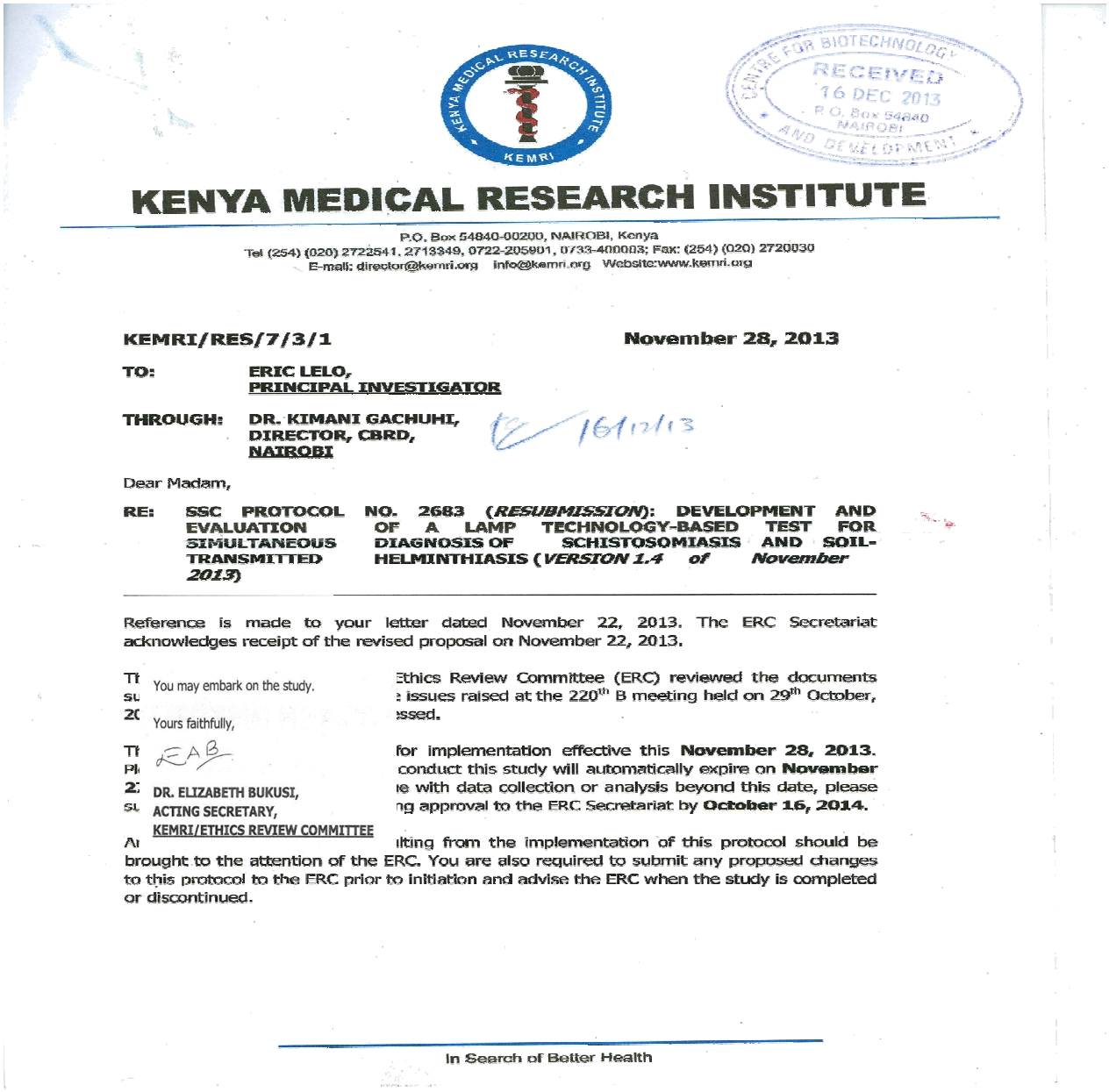
_____

Name of the Person Obtaining Consent and Signature (or Thumb Print)

_____

Name and Signature (or Thumb Print) of the Witness

Appendix 3: Ethical clearance form.



KENYA MEDICAL RESEARCH INSTITUTE

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E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

CENTRE FOR BIOTECHNOLOGY RESEARCH AND DEVELOPMENT
RECEIVED
16 DEC 2013
P.O. Box 54840
NAIROBI

KEMRI/RES/7/3/1 **November 28, 2013**

TO: **ERIC LELO,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. KIMANI GACHUHI,**
DIRECTOR, CBRD,
NAIROBI

16/12/13

Dear Madam,

RE: **SSC PROTOCOL NO. 2683 (*RESUBMISSION*): DEVELOPMENT AND EVALUATION OF A LAMP TECHNOLOGY-BASED TEST FOR SIMULTANEOUS DIAGNOSIS OF SCHISTOSOMIASIS AND SOIL-TRANSMITTED HELMINTHIASIS (*VERSION 1.4 of November 2013*)**

Reference is made to your letter dated November 22, 2013. The ERC Secretariat acknowledges receipt of the revised proposal on November 22, 2013.

Tl You may embark on the study. Ethics Review Committee (ERC) reviewed the documents su issues raised at the 220th B meeting held on 29th October, 2(ssed.

Yours faithfully,

Tl for implementation effective this **November 28, 2013.** Pl conduct this study will automatically expire on **November** 2: ie with data collection or analysis beyond this date, please su ng approval to the ERC Secretariat by **October 16, 2014.**

EAB

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

Al ilting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the ERC prior to initiation and advise the ERC when the study is completed or discontinued.

In Search of Better Health

Appendix 4: Publication from this study.

Robert Muriuki Mugambi, Eric L.Agola, Ibrahim N. Mwangi, Johnson Kinyua, Esther Andia Shiraho and Gerald M. Mkoji (2015). Development and evaluation of a Loop Mediated Isothermal Amplification (LAMP) technique for the detection of hookworm (*Necator americanus*) infection in feacal samples. *Parasites & Vectors* (**8**), 574