APPLICATION OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) FOR DIAGNOSIS OF *ASCARIS LUMBRICOIDES* IN FECAL SAMPLES

ESTHER ANDIA SHIRAHO

MASTER OF SCIENCE

(Molecular Medicine)

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY

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Development of a Loop-Mediated Isothermal Amplification (LAMP) for Diagnosis of *Ascaris lumbricoides* in Fecal Samples

Esther Andia Shiraho

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DECLARATION

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

Signature ……. Date ……

Esther Andia Shiraho

This thesis has been submitted for examination with the approval of the following supervisors:

Signature ……. Date ……

Dr. Eric Lelo Agola, PhD

KEMRI, Kenya

Signature ……. Date ……

Prof. Venny C.S. Nyambati, PhD (deceased)

JKUAT, Kenya

Signature ……. Date ……

Dr. Gerald M. Mkoji, PhD

KEMRI, Kenya

DEDICATION

To three wonderful women in my life; my Mom Elizabeth Khadushi, Sisters Bephine Nyawera and Everlyne Khalai, and to my late Dad Richard Shiraho.

Thus far, the Lord has helped us.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

B3	Backward outer primer
BIP	Backward Inner Primer
Bsm	*Bacillus smithii*
Bst	*Bacilus stearothermophilus*
DNA	Deoxyribose nucleic acid
dsDNA	Double stranded Deoxyribose nucleic acid
dNTPs	Deoxynucleotide triphosphates
F3	Forward outer primer
FIP	Forward Inner Primer
ITS	Internal transcribed spacer
LAMP	Loop mediated isothermal amplification
MDA	Mass drug administration
NTDs	Neglected Tropical Diseases
PCR	Polymerase chain Reaction
STH	Soil transmitted Helminthes
TTTT	Spacer sequence at the hinge region of FIP and BIP primers
WHO	World Health Organization

ABSTRACT

Ascaris lumbricoides is a nematode parasite that causes ascariasis in humans. It is also considered among the neglected tropical diseases. Diagnosis relies mainly on microscopy-based methods which are laborious, require high expertise and limited by low sensitivity. This study sought to develop a loop-mediated isothermal amplification (LAMP) for diagnosis of ascariasis in fecal samples. Primer Explorer V4 software was used to design primers based on the first internal transcribed (ITS-1) spacer region of the ribosomal DNA. Four consensus sets of primers, which successfully identified and amplified 6 regions of the target sequence, were adopted due to their reliability. *Ascaris* adult and ova were obtained from naturally infected school children, whose parents/guardians consented their participation in the study. Genomic DNA was extracted using HotShot alkaline lysis method after harvesting *Ascaris* ova from feces using the modified Wisconsin floatation method, and amplified by LAMP at 63^{o}C for 45 minutes. Blinded samples were used as clinical samples to test for LAMP after direct DNA extraction using the QIAmp Fast DNA stool minikit. LAMP products were visualized by naked eyes on addition of SYBR green dye, a DNA intercalating orange dye that turns green if amplification is positive. This was additionally confirmed on agarose gel that produced ladder-like bands due to inverted repeats of the target sequence on the same strand. LAMP technique developed successfully and reliably detected *Ascaris* DNA from a single ovum and in fecal samples. The assay specifically detected *Ascaris* DNA without amplifying DNA from ova of hookworm, *Trichuris trichiura* and *Schistosoma mansoni*, parasites which commonly co-occur with *A. lumbricoides* in feces. The developed LAMP assay has great potential for use in ascariasis diagnosis at the point of care and in low infection intensity situation that are characterized by control and elimination campaigns.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Ascaris lumbricoides causes soil-transmitted helminthiases (STHs) worldwide and is a major public health problem, considered as one of the neglected tropical diseases, and currently targeted for elimination (Hotez and Kamath, 2009). Contemporary global estimates of ascariasis in 2010 was found to be 771.7 – 891.6 million, according to a mathematical modeling approach by Pullan and colleagues (2014). Infection with *Ascari* is through the fecal-oral route *(*Dold and Holland, 2011). The epidemiological factors responsible for ascariasis and other soil transmitted helminth infections include poor sanitation and hygiene, inadequate water supplies and poor health care systems (Gordon *et al.,* 2011). Increased travel of *Ascaris* and other STH infections has become more common in non-endemic areas due to increased travel and migration (Lamberton and Jourdan, 2015).

World Health Organization (WHO) recommends diagnosis of *Ascaris* by examining fecal sample under a microscope for the parasite ova using Kato-Katz technique (WHO, 2010). Microscopy-based diagnostic tests suffer from low sensitivity, are cumbersome to use, require expertise to perform, and lack throughput capability (Phuphisut *et al.*, 2014). Sero-diagnostic tests exist but rarely used for routine detection of *Ascaris* infections because they are prone to false positive results; especially due to cross-reactivity of anti-*Ascaris* antibodies with epitopes of other infections and helminths (Knopp *et al.,* 2008, Haswell-Elkins *et al*., 1992). Reliable diagnostic tests such as molecular based techniques are needed for infection diagnosis, monitoring effectiveness of chemotherapy, and for disease surveillance post-elimination of ascariasis (Hotez *et al.*, 2007). Polymerase chain reaction, real-time quantitative PCR (qPCR) and multiplex assays are highly sensitive, specific and rapid but are costly to implement in disease endemic setting due to the limited resource (Lamberton and Jourdan, 2015) and need for expensive equipment. Loop Mediated Isothermal Amplification (LAMP), is a powerful

alternative to both conventional parasitological tests and molecular techniques, and is being considered for exploitation for diagnosis of diseases including human ascariasis.

1.2 Statement of the problem

Soil-transmitted helminthiases (STHs) are currently targeted for elimination through mass drug administration programmes by the World Health Organization. These programmes have been very successful in the reduction of infection intensities in disease endemic areas. Current routine diagnosis of ascariasis relies on microscopic examination of fecal samples prepared by the Kato-Katz or formol-ether concentration methods of parasite ova detection. Microscopy however is not sensitive enough to detect low-level infections leading to underestimation of prevalence and hampering accurate measure of success of disease elimination programmes. Moreover, the parasitological tests require experienced microscopists to make accurate diagnosis. Currently, there are ten molecular techniques that utilize polymerase chain reaction method, that have been used for diagnosis of nematode infections having high specificity and sensitivity advantage over traditional diagnostic methods. However, they have setbacks such as lengthy run timers; high cost of equipment and reagents; optimization of reactions can be time consuming especially for multiplex PCR and results need to be visualized on a gel.

1.3 Justification of the study

Accurate, rapid and reliable methods are necessary for diagnosis of ascariasis. Loop-mediated isothermal amplification (LAMP) technique is being evaluated as a diagnostic tool and currently being exploited for diagnosis of soil-transmitted helminthiases, where appropriate diagnostic tests are still needed to support disease prevention and control efforts, with the renewed interest and focus on the control of neglected tropical diseases.

1.4 Research Questions

1. What is the suitable DNA target that could be used for development of a LAMP-based diagnostic test for ascariasis.
2. Could the LAMP technique be optimized for future adaptation in the diagnosis of ascariasis in fecal samples?
3. How does LAMP technique compare with the gold standard in detecting ascariasis in fecal samples?

1.5 Objectives

1.5.1 General Objective:

To apply a simple, reliable, and inexpensive molecular diagnostic test based on LAMP technique for detection of *Ascaris lumbricoides* infection in human fecal samples.

1.5.2 Specific Objectives:

1. To determine a suitable DNA target for the detection of *A. lumbricoides* infection in human fecal samples in a LAMP assay.
2. To optimize conditions for the LAMP assay for detection of *A. lumbricoides* infection in human fecal samples.
3. To evaluate the developed LAMP assay for its ability to detect *A. lumbricoides* infection in fecal samples in comparison

CHAPTER TWO

LITERATURE REVIEW

2.1 Ascariasis as a public health concern

Ascaris lumbricoides is an intestinal nematode belonging to the family Ascarididae and is the etiological agent for ascariasis, which infects humans (Dangana *et al.*, 2011). It is estimated that 1.2 billion people are affected with *A. lumbricoides* (Crompton, 1999). The adults are also often infected with *A. lumbricoides* but prevalence and infection intensities tend to be much lower than in children (Dangana, 2011). There has not been any particular study to look at the overall burden of ascariasis either in Kenya or Africa as a whole, but several studies in particular areas do exist. In Enugu Metropolis of Nigeria, Chijioke and colleagues (2011) placed the prevalence of ascariasis at 19.1%. In Mbeya region of Tanzania, ascariasis prevalence was found to be on average 6.9% with specific sites having 25.1 % (Kyela) and Isongole (16.9%) (Steffen *et. al*, 2014). In Kenya multiple studies show the same trend of high ascariasis prevalence among other STHs; in western Kenya following a school-based deworming exercise – 17% (Garn *et al*., 2016); Migori – 17% (Ng'ang'a *et al*, 2016); whereas Mulambalah and Ruto (2016) after a cross-sectional school- based study in Nandi found ascariasis to be the most prevalence, accounting for 42-74% STH infections. Adults get infected with ascariasis, but school-aged children bear the greatest burden, and a recent observation suggests that pre-school aged children are possible reservoirs of STH infection (Musuva *et al*., 2017).

The epidemiological factors responsible for ascariasis and other soil transmitted helminth infections include poor sanitation and hygiene, inadequate water supplies and poor health care systems (Gordon *et al*., 2011). The strategy recommended by WHO (2006) to control morbidity from Ascariasis and other STHs involves the periodic administration of anthelminthic medicines, mainly single dose 400 mg albendazole and 500 mg mebedazole to the population at risk of the disease.

Ascaris lumbricoides has a simple, direct life cycle that involves fecal-oral transmission of the infective eggs (Dold and Holland, 2010). Humans become infected when they

ingest a fully embryonated fertilized egg. The adult *A. lumbricoides* inhabit the small intestine where the male and female mate and produce eggs that are passed out into the environment with feces (Dold and Holland, 2010).

2.2 Diagnosis of Ascariasis

In many pathogenic helminth infections as with ascariasis, disease presentation is ordinarily due to physical factors related to the location of the worms and their size or number. The standard method for diagnosing ascariasis is by identifying *Ascaris* eggs in stool sample using a microscope (Seo, 1983). Because eggs may be difficult to find in light infections, WHO (2002) recommended the Kato-Katz thick smear as the standard quantitative diagnostic technique. Other methods include; FLOTAC technique (Cringoli, 2006), zinc sulfate flotation, direct observation of the worm incase passed out in vomit or feces and formol-ether concentration, direct fecal smear, McMaster technique (WHO, 2008) under a microscope for the parasite ova. Eosinophilia is quite common with helminth infection, thus complete blood count can be conducted in case helminthiasis is suspected. The problem is other medical conditions such as allergies, hay fever, and asthma also cause eosinophilia.

Yamashita *et al.,* (2013) suggested that capsule endoscopy could serve as a crucial, non-invasive diagnostic tool for *A. lumbricoides* infection, especially when other diagnostic methods have failed to detect the parasite. They reported a case of *A. lumbricoides* infection that resulted in intestinal obstruction at the level of the ileum. In this case, both stool sample examination and open surgery failed to indicate the presence of ascariasis, and the cause of obstruction was only revealed by capsule endoscopy.

Although microscopy-based diagnostic tests have value in infection diagnosis, in disease control or elimination campaigns, they suffer from low sensitivity, are cumbersome to use, require expertise to perform, lack throughput capability, and generally, they may not be useful in detecting low level infections, for monitoring success or failure of chemotherapy or for disease surveillance in disease elimination campaigns, hence,

neither are they adaptable for use in field settings, or at the point-of-care (Hotez *et al.,* 2007, Tarafder *et al*., 2010).

Sero-diagnostic tests exist but rarely used for routine detection of *Ascaris* infections. Antibody production by *A. lumbricoides* varies with exposure and intensity of infection, especially in high-endemic areas (Haswell-Elkins *et al*., 1992; Hagel *et al*., 2008). Other challenges facing serological diagnosis include: they are more invasive, rely on detection of antibodies that may persist after drug treatment and cure; cross-reactivity of anti-Ascaris antibodies with epitopes of other infections and helminths (Knopp *et al.,* 2008, King *et al*., 2005 Haswell-Elkins *et al*., 1992).

2.2.1 Molecular Diagnosis of Ascariasis

The advent of genomic sequencing and the wealth of data generated have markedly increased the feasibility of developing polymerase chain reaction methods as diagnostic tools for helminth parasites (Gordon *et al.,* 2011). Molecular based tests utilizing PCR are powerful tools, usually with high sensitivity and specificity, depending on the genetic markers used (Gordon *et al.,* 2011). Rapid advances are being made, resulting in reduced costs and improved techniques such as real-time quantitative PCR (qPCR), Copro PCR, blood serum PCR, conventional PCR, nested PCR, multiplex PCR, real time PCR, multiplex RT-PCR, RFLP PCR and DNA probes. Table 2.1 shows molecular methods that have been used for diagnosis of parasitic nematodes. Although molecular diagnostic tools are powerful alternatives to conventional parasitological tests offering greater sensitivity, specificity and reliability, they have setbacks such as lengthy run timers; high cost of equipment and reagents; optimization of reactions can be time consuming especially for multiplex PCR and results need to be visualized on a gel hence not adaptable for use in field setting. There is need to develop a gold standard method that combines good sensitivity with quantitative performance, low cost, and easy-to-lean technique. Loop mediated isothermal amplification (LAMP) is one such promising technique.

Table 2.1: Molecular techniques applied for diagnosis of nematode infection

PCR-based and other DNA amplification techniques applied for diagnosis of human nematode infection. (Gordon et al., 2011)

Parasitic nematode	Copro PCR	Tissue PCR	Blood/ Serum PCR	Conventional PCR	Nested PCR	Multiplex PCR	Real-time PCR	Multiplex real-time PCR	RFLP PCR	DNA Probes	LAMP
Trichuris trichiura				√		√					
Ancylostoma duodenale	√			√		√	√	√		√	
Necator americanus	√			√		√	√	√		√	
Onchocerca volvulus				√	√		√				
Wuchereria bancrofti			√	√	√	√	√		√		
Toxocara canis	√			√							√
Toxocara cati	√			√							√
Ascaris lumbricoides	√			√		√		√		√	
Strongyloides stercoralis	√			√	√	√	√	√			

2.3 The Loop Mediated Amplification (LAMP) method.

Loop-mediated isothermal amplification was solely developed by Eiken Chemical Co., Ltd., Tokyo, Japan in 1998, is an innovative amplification technique that amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions, without a significant influence of the co-presence of non-target DNA (Macuhova *et al.*, 2010). It was first described by Notomi *et al.*, (2000), but has now found wide application as a diagnostic tool for a wide variety of human, animal and plant diseases caused by protozoans, bacteria, or viruses (Li *et al*., 2013, Obura *et al*., 2011, Wang *et al*., 2012, Yamazaki *et al*., 2011). The technique uses a thermo-stable DNA polymerase called *Bst* or *Bsm* DNA polymerase which has displacement activity at an average constant temperature of 65° C. In addition, LAMP uses four primers, these are a set of 2 inner primers (forward (FIP) and backward (BIP)) and outer primers (forward (F3) and backward (B3)), which recognize six sections of the target gene (Einken, 2013). It has high specificity, attributable to recognition of 6 distinct regions on the target gene by the four primers. This partly alleviates the general problem of backgrounds associated with all nucleic-acid amplification methods. For instance, LAMP is capable of rapidly amplifying target nucleic acids with greater sensitivity, specificity, efficiency and rapidity (Karanis *et al.,* 2007), without a significant influence of the co-presence of non-target DNA (Macuhova *et al.,* 2010). Furthermore, LAMP-based assays require relatively simple and inexpensive equipment such as an incubator, water bath and a heat block which can provide constant temperature required for the DNA amplification, and therefore can easily be applied at the point of care, even under field conditions (Karanis *et al.,* 2007). Amplification and detection of the genes can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase and substrates at a constant temperature using a simple water bath or heat block (Notomi *et al.,* 2000). Products of LAMP reaction when visualized on gel electrophoresis comprise alternately inverted repeats of the target sequence on the same strand (Eiken Chemical Co., Ltd., Tokyo, Japan). Generally, LAMP products can be visualized by the naked eye for colour change on addition of SYBR green dye, which is orange but turns green if positive. Recent studies have demonstrated that LAMP is more sensitive and specific

than conventional polymerase chain reaction (Ai *et al., 2010,* Xu *et al., 2010*). Additional benefits include speed of the assay, shorter run time, and ease of training personnel lacking a background in molecular techniques, and throughput capability.

2.3.1. Basic Principles of the LAMP Technique

The primers are usually generated by online software, Primer Explorer version 3 or 4 for identification of suitable DNA target. Four sets of primers BIP, FIP, B3, F3 (Backward Inner Primer, Forward Inward Primer, Backward outer primer and Forward outer primer respectively) will be generated having the ability to recognize 6 regions of the target gene that is F3c, F2c and F1c of the 3' end and B1, B2 and B3 regions of the 5' end. The inward primers comprise of two sets of sequence separate by a TTTT sequence at the hinge region; F1c and F2 for FIP, and B1c and B2 for BIP. The software ensures that GC and AT content are 50-60 % and 40 – 50 % respectively.

The LAMP master mix; target DNA, *Bst/Bsm* DNA polymerase, *Bst/Bsm* buffer, primers (BIP, FIP, B3 & F3), and LAMP reaction buffer (TrisHCl, KCl, $MgSO_4$, $(NH_4)_2SO_4$, Tween 20 and dNTPs), placed in a water bath or heating block at a constant temperature ranging between 60 - 65^o C. DNA amplification proceeds as described below.

At 65^o C, double stranded DNA is in dynamic equilibrium that enables LAMP primers to anneal to complementary sequence of double stranded target DNA. DNA synthesis is initiated by *Bst* or *Bsm* DNA polymerase starting from the 3' end of F2 region of FIP. F3 primer anneals to F3c region outside FIP on the target DNA, initiating strand displacement; releasing FIP linked complementary strand. A dsDNA results from DNA strand synthesized from F3 primer of the template DNA strand releasing a single strand FIP linked complementary strand. This single strand forms a stem loop structure at 5' end because of the complementary F1c and F1 regions which serves as the template for BIP initiated DNA synthesis and subsequent B3 primed strand displacement.

Backward inner primer anneals to the looped FIP linked strand and complementary DNA synthesized from 3' end. Eventually, the looped DNA structure becomes linear. Backward-3 primer anneals to the outside of the BIP and through *Bst/Bsm* DNA polymerase, starting at 3' end, DNA synthesized from the BIP is displaced and released as single strand before DNA synthesis from the B3 primer. This process results in production of dsDNA.

The above process occurs concurrently at a thermo-stable temperature, ultimately resulting in formation of various sized structures consisting of alternately inverted repeats of the target sequence on the same strand.

2.3.2 Improvement of the LAMP Technique

2.3.2.1 Use of loop primers

In 2002, Nagamine and colleagues were able to prove that on addition of loop primers (forward and backward) to the four existing primers, LAMP reaction time was significantly reduced. Loop primers hybridize to the stem-loops, except for the loops that are hybridized by the inner primers, and prime strand displacement DNA synthesis. Although both inner and loop primers react via the loops, they do so by different mechanism. The LAMP reaction time was accelerated, and original time cut by half (Nagamine *et al.*, 2002).

2.3.2.2 The TTTT sequence at the hinge region of inner primers

When Notomi and colleagues in their first publication designed primers (2000), a TTTT spacer sequence was inserted between F1c and F2, and B1c and B2 of FIP and BIP primers respectively. The current use of the web based programme primer explorer for designing LAMP primers tends to omit the TTTT sequence instead replacing it with a dash (Torres *et al.*, 2011).

2.3.2.3 Addition of Betaine

Different authors have had contradicting reports, with some indicating no effect on LAMP reaction with or without addition of betaine (Zhou *et al.,* 2014). Njiru in his 2011 study indicated that efficacy of LAMP amplification was enhanced using betaine with a concentration of 0.8 M.

2.3.3 Detection of LAMP Products

Several methods exist that detect LAMP amplification product. These include:-

(a) Turbidity measurement using a turbidimeter. The turbidity is usually caused by accumulation of magnesium pyrophosphate, which can also be seen by the naked eyes.

(b) Fluorescence caused by unquenching of calcein during reaction can be measured using a fluorescence detector.

Gel electrophoresis is mostly used to confirm amplification of DNA that usually appear as ladder like bands (Notomi *et al.,* 2000) due to inverted repeats of the target sequence on the same strand (Eiken Chemical Co., Ltd., Tokyo, Japan).

(c) Staining with SYBR green dye and observe for colour change with the naked eyes (Notomi *et al.,* 2000).

2.4 Loop mediated isothermal amplification as a diagnostic tool for pathogens

The LAMP provides a powerful way to diagnose diseases, and has already been widely adopted and further developed for detection of various pathogens including bacteria, plants, viruses and protozoans, and detecting transgenes in genetically modified organisms (Dinggand *et al*, 2014, Gurinder *s,* 2013). It has also been applied in diagnosis of human parasitic infections caused by cestodes such as *Taenia asiastica, T. saginata* and *T. solium* (Ai *et al.,* 2010; Nkouawa *et al.,* 2009, 2010), whose DNA were detected in human faecal samples. It has also been used for detection of *Schistosoma haematobium* and *S. mansoni* (Abbasi *et al.,* 2010; Abbasi *et al.,* 2010); for detection of *Paragonimus westermani* in sputum and pleural (Chen *et al.,* 2011). So far, Toxocara canis and T. cati are the only nematodes that have been detected using LAMP technique

(Macuhova *et al.,* 2010), which gave leeway to using the same technique for diagnosis of *Ascaris*.

2.5 Diagnosis of ascariasis using LAMP technique

A study conducted by Blouin, (2000) and Zhu *et al.*, (1999) sort to look at the mitochondrial DNA and internal transcribed spacer regions of ribosomal DNA of nematodes. He observed that ITS-1 and ITS-2 sequences were commonly used as markers for discrimination among nematode species (Powers *et al*., 1997 & Gasser and Newton, 2000). Internal transcribed spacer region was also found to be slow at evolving (conserved among and between species), compared to mtDNA, hence more useful to differentiate between closely related species (Morgan and Blaire, 1998, Pecson *et. al*, 2006). In addition, ITS regions have variable nuclear loci, and that most universal primers based on ITS regions are available and work with most nematodes. (Blouin, 2002); and concluded that ITS was an excellent tool for DNA diagnostics owing to its lower level of intraspecific polymorphism. Mitochondrial DNA is argued to be the best choice for applications in which one is using sequence data on small numbers of individuals to search for potential cryptic species.

For this experiment, I used the ITS-1 sequence of the ribosomal DNA as the target, being the first attempt to use LAMP for diagnosis of ascariasis.

Ascariasis is a neglected tropical disease that is currently targeted for control and elimination using the mass drug administration (MDA), also referred to as preventive chemotherapy to reduce morbidity (Hotez *et al.,* 2007). Reliable diagnostic tests are therefore needed not only for infection diagnosis, but also for monitoring effectiveness of chemotherapy or for disease surveillance post-elimination. This technique that I have developed based on LAMP promises to be a powerful molecular-based diagnostic tool as an alternative to conventional parasitological tests; offering greater sensitivity, specificity and reliability (Gordon *et al.*, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was conducted at the Centre for Biotechnology Research and Development (CBRD) at Kenya Medical Research Institute (KEMRI) after collection of samples in Mswambweni Sub-county of Kwale County in south-coast of Kenya. The site for sample collection was chosen due to the presence of Madrasa schools; Islamic institutions/schools some which exclusively teach Islamic educations whereas others are integrated with formal education under the Ministry of Education curriculum.

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3.2 Study design

This is a cross-sectional laboratory-based study design; that applied LAMP technique for diagnosis of ascariasis in fecal samples obtained from school-aged children with natural infection of *Ascaris lumbricoides*

3.3 Sampling method and sample size

I used convenience sampling, a nonprobability sampling technique (Etikan *et al*., 2016), for I was only interested in getting enough STH positive samples for use in development and evaluation of LAMP technique.

3.4 Study approvals and ethical considerations

This study was approved through the Scientific and Ethics Review Unit (SERU) of the Kenya Medical Research Institute. I adhered to ethical requirements such that all children found to be infected with *A. lumbricoides* and/or other soil-transmitted helminths received the WHO recommended standard treatment for STHs of 400mg albendazole (WHO, 2006) in a single dose administered by a qualified and registered clinical officer. Fecal 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.

3.5 Fecal sample collection and parasitological examination

Fecal samples were collected from 581 children aged 6-12 years, attending the following Madrasa schools; Kingwede, Magaoni, Nganja, Mwisho wa Shamba, Marigiza, Majikuko, Munje, Mwendo wa Bure, Bodo, Fahamuni, Najaha, Shirazi, Vunduguni, Nyumba sita, Mchinjirini, Munje Pwani, and Mlalani within Msambweni sub-county. Only those whose parents/guardians had consented to their participation in this study took part in the exercise. Three parasite species, namely, *A. lumbricoides*, hookworm and *T. trichiura* were detected by microscopy on the Kato-Katz smears. From our collected samples, the overall prevalence of the three intestinal helminth infections among the children examined was 40% with prevalence of *Ascaris* infection standing at 7.9%. Ova of *S. mansoni* were recovered from lab infected mice donated from an ongoing approved study within the CBRD- KEMRI. These three provided DNA for specificity testing of the LAMP assay.

A single adult *A. lumbricoides* worm was recovered from a fecal sample of a child who had received 400 mg albendazole. The specimen was preserved in 95% ethanol and later used as positive control for the LAMP assay.

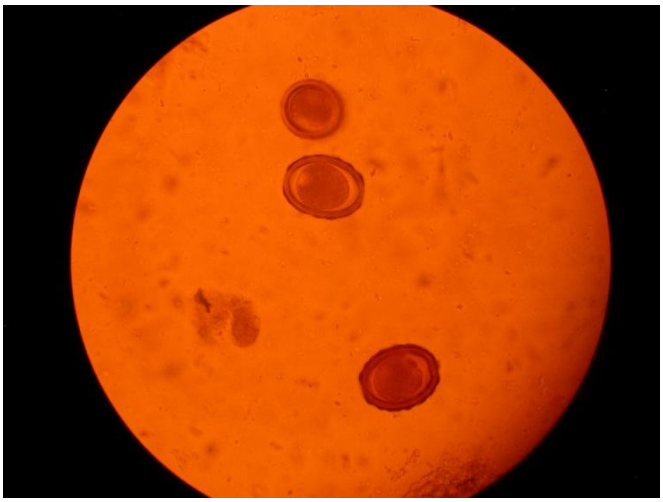

Figure 3.1: *Ascaris lumbricoides* ova.

A microscope slide of *A. lumbricoides* ova observed at X400 magnification isolated from fecal sample using Modified Wisconsin Floatation method.

3.6 Fecal sample collections, and isolation of *Ascaris* ova for DNA extraction

Ascaris ova needed for DNA extraction and optimization of the LAMP assay were isolated from naturally infected children after receiving consent from parents or guardians. Each participating child provided a single fecal sample in a capped plastic polypot, which were then transported back to the field laboratory in an ice box within 2 hr of collection. Duplicate Kato-Katz microscope slides were prepared as described by Barbosa and colleagues (Barbosa *et al.*, 2017) and examined under a compound microscope at X400 magnification by two experienced microscopists. Also, 5% of the total number of slides was read by a third microscopist for quality control purposes. *A. lumbricoides* ova positive samples were used to isolate ova for DNA extraction which was used for optimizing the LAMP technique.

Ova o*f A. lumbricoides* were isolated from individual fecal samples using the modified Wisconsin's sugar floatation method (Egwang and Slocombe, 1982). Briefly, Sheather's sugar solution with a specific gravity of 1.27 was prepared by mixing 455gm table sugar with 355ml distilled water and boiled to dissolve the sugar. Approximately 6gm of fecal

sample was then placed in a 3.5inch diameter petri dish and 30ml of the Sheather's solution added. The mixture was stirred into a homogenate using a wooden tongue depressor, and filtered through a layered wet cheese-cloth into a 50ml centrifuge tube. The filtrate was centrifuged at 3000rpm for 10 min, and the solution was left to stand for 10 min to allow the parasite eggs to float. The presence of parasite ova was confirmed under the microscope by placing a cover slip over the solution and transferring it onto a microscope slide (Egwang and Slocombe, 1982). Approximately 5ml of the supernatant was transferred into a 15ml centrifuge tube, and 2 volumes of water added to dilute the sugar solution, consequently reducing the specific gravity. This allowed the eggs to settle at the bottom after a 3 min spin in the centrifuge at 3000rpm. The resulting pellet was re-suspended in 2 ml distilled water, mixed by vortexing and then transferred into 1.5ml centrifuge tubes.

3.7 Literature search for suitable DNA target for diagnosis of Ascariasis

After considering several DNA targets for their suitability in providing diagnostic markers for human ascariasis in a LAMP assay, the first internal transcribed spacer (ITS-1) region of the ribosomal DNA was selected as the most suitable target for primer design.

Table 3.1: Adult *Ascaris lumbricoides* first internal transcribed spacer ribosomal DNA gene sequence 1..446

1 atcgagcaga aaaaaaaaag tctccgaacg tgcacataag tactatttgc gcgtatacgt
61 gagccacata gtaaattgca cacaaatgtg gtgatgtaat agcagtcggc ggtttctttt
121 tttttggcgg acaattgcat gcgatttgct atgtgttgag ggagaatagg tggcatgttg
181 ggcttgttag aaaggcatgc stagcgctta ttttcccgct atttcgtaac aacggtgtcc
241 attttggcgt ctacgcttca ccgagctatc gcctggaccg tcggtagcga tgaaaggtgg
301 agagaaagct cctcgtttcg rgtcgagtag actcaatgag cctcagcttg gaggccgcca
361 aaactcaaaa aacacaatca cttttgaaaa tctattctaa tgaaagatgc taaattttgt
421 ttagtatctt cgaattgtaa gatgaa

3.8 Designing LAMP Primers

Primer Explorer V4 software (http://primerexplorer.jp/elamp4.0.0) was used for primer design (Thekisoe *et al*., 2010). Three sets of primers were generated (Table 3.1), and analyzed by Basic Local Alignment Search Tool (BLAST) (*blast.ncbi.nlm.nih.gov/Blast.cgi) t*o verify their suitability. Nucleotide BLAST of F3, B3, FIP and BIP for all the three primer sets revealed the relatedness of the primers to the sequence of *Ascaris* found in the NCBI database. The third primer set was chosen for subsequent experiments as it had a score > 40 and an E-value of 0.26. The other two primer sets had E-value of over 4.0 and a score of less than 40. The higher the score and the closer the E-value is to zero, the higher the statistical significance of relatedness to *A. lumbricoides,* from GenBank sequence accession number AJ000895 (https://www.ncbi.nlm.nih.gov/nuccore/AJ000895) were. The primers were manufactured by Bioneer Singapore, through BecA-ILRI Hub Services – SegoliP laboratories Nairobi, Kenya. After several trials, only one set of primers for the ITS-1 region (Table 3.1) was chosen having FIP, BIP, F3 and B3 (Table 3.2).

Table 3.2: Primers generated for LAMP from first internal transcribed spacer ribosomal DNA of *Ascaris lumbricoides* adult worm

The 3rd highlighted set was selected having the lowest E-value after BLAST

Primer Set		Sequence, 5'→3'	
1st	F3	CGGTTTCTTTTTTTTTGGCG	
	B3	GAGGAGCTTTCTCTCCAC	
	FIP	TCTAACAAGCCCAACATGCC-CAATTGCATGCGATTTGCT	
	BIP	CCGCTATTTCGTAACAACGGT-CTTTCATCGCTACCGACG	
2nd	F3	GCCACATAGTAAATTGCACAC	
	B3	CGTAGACGCCAAAATGGA	
	FIP	CAAATCGCATGCAATTGTCCG-AAATGTGGTGATGTAATAGCAG	
	BIP	ATGTGTTGAGGGAGAATAGGTGG-TTGTTACGAAATAGCGGGAA	
3rd	F3	CTTGTTAGAAAGGCATGCTAG	(Tm 56.12)
	B3	GTGTTTTTTGAGTTTTGGCG	(Tm 56.08)
	FIP	TAGCTCGGTGAAGCGTAGAC-CTTATTTTCCCGCTATTTCGTA	(Tm 55.86 - 60.75)
	BIP	GACCGTCGGTAGCGATGAAA-GCTCATTGAGTCTACTCGA	(Tm 55.20 - 61.62)
3rd modified inward primers	FIP	TAGCTCGGTGAAGCGTAGAC-**TTTT**-CTTATTTTCCCGCTATTTCGTA	
	BIP	GACCGTCGGTAGCGATGAAA-**TTTT**-GCTCATTGAGTCTACTCGA	

Table 3.3: Position of the primers on the adult *Ascaris lumbricoides* gene

The 446 long gene sequence, and the table shows the exact position of the primer sequence.

Primer		Range / Position
F3		183 - 200
B3		356 - 375
FIP	F1c	249 - 268
	F2	207 - 228
BIP	B1c	276 - 295
	B2	323 - 341

3.9 DNA Extraction

DNA was extracted from intact eggs of *A. lumbricoides*, as well as from an adult worm using the modified HotShot method (Blouin, 2002). Briefly, 1, 5, 10, 15, 20 and 25 *Ascaris* eggs were placed into 6 separate PCR tubes respectively, each containing 30 µL of lysis reagent (NaOH, EDTA, distilled water), and incubated at 95^{o}C for 1hr. An equal volume of neutralizing reagent (TrisHCl, distilled water) was added to each of the tubes at the end of the incubation period (Blouin, 2002). The DNA concentration was determined for each tube to estimate the amount of DNA available for the different egg counts using the NanoDrop 2000 (Thermo Scientific, Wilmington DE, USA). To extract DNA from an ethanol-preserved adult *A. lumbricoides* worm, the specimen was cut up into tiny pieces each measuring ~1mm. These were then placed in a petri dish containing distilled water and soaked for 1hr to remove ethanol traces. Each piece was then transferred into a 200µL tube containing 40 µL of lysis reagent and incubated at 95^{o}C for 1 hr, followed by addition of equal volume of neutralizing reagent (TrisHCl, distilled water) to end up with DNA solution in TE buffer (Blouin, 2002). To evaluate the specificity of the developed LAMP test, three other parasites ova were used, namely; hookworm, *Schistosoma mansoni* and *Trichuris trichiura.* These are fecal-based helminth parasites that commonly co-occur with *A. lumbricoides*. DNA from ova of *S.*

mansoni and *T. trichiura* was extracted as described above using the HotShot method (Blouin, 2002).

All the collected fecal samples were not fixed in either ethanol or formaldehyde, the commonly used fixing agents, as they were to be used for molecular work. Unlike ova from *A. lumbricoides*, *T. trichiura* and *S. mansoni*, hookworm ova tend to hatch so fast, hence I was unable to separate the same from fecal matter. For this reason, hookworm ova DNA was extracted directly using the QIAmp Fast DNA stool Mini Kit (QIAGEN, Hilden, Germany) as described by the manufacturer. 200 g of stool was weighed and placed in a 2 ml tube. 1 ml of EX buffer was added to it and vortexed for 1 min. The homogenized solution was heated for 5 minutes at 95°C, followed by 15 sec of vortexing. 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, added 15 µl of proteinase K followed by 200 µl of the supernatant from the centrifuged stool solution and finally 200 µl of buffer AL. The mixture was vortexed for 15 sec followed by incubation at 70°C for 10 minutes. 200 µl of absolute ethanol was added and the followed by 10 sec of vortex. 600µl of the resulting lysate solution was pippeted into a spin column and centrifuged for 1 min at 14,000 rpm. The filtrate was then discarded. To the same spin column 500µl of buffer1 (AW1) was added and centrifuged for 1 min 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 spin column was replaced on an empty tube for another dry spin at 14,000 rpm for 3 min to remove any residual buffers. The spin column was placed onto a clean 1.5 ml tube and 200µl of DNA elution buffer added, and incubated at room temperature for 1 min. This was span for 1 min at 14,000 rpm to elute the DNA.

Table 3.4: Concentration of DNA from parasite ova

No. of *Ascaris* ova from which DNA was extracted	Parasite ova DNA concentration in ng/µL
1	**10.8**
5	**13.1**
10	**19**
15	**30.5**
20	**38.1**
25	**44.2**

3.10 Optimization of the LAMP reaction conditions

I used the DNA from adult worm as positive control and serial diluted the concentration to 5 ng/µL I also diluted the extracted DNA concentration of the single ova in a 1:2 fold and used 5 ng/µL for use in optimization. I experimented with several ratios of ITS-1 LAMP based primers; specifically, ratios of the inner to outer primers as follows; 2:1; 4:1; 6:1 and 8:1 (Su *et al*., 2016, Notomi *et al*., 2000, Li *et al*, 2013, Obura *et al*., 2011, Yamazaki *et al*., 2011, Karanis *et al*., 2007, Macuhova *et al*., 2010 and Ai *et al*., 2010). The LAMP reaction sensitivity, effectiveness and repeatability greatly relies on the proper ratio of inner to outer primer. Secondly, several incubation time ranges between 15min – 2 hr with intervals of 15 min were tested for a constant amount of DNA concentration and fixed primer ration. Thirdly, the ideal incubation temperatures for each incubation time were optimized. The temperatures tested ranged from 56^{o}C to 63^{o}C based on the base composition of each primer and the recommended melting temperature (T_m) 56 ^{o}C of each primer (Notomi *et al*., 2000, Li *et al*, 2013, Obura *et al*., 2011, Yamazaki *et al*., 2011, Karanis *et al*., 2007, Macuhova *et al*., 2010 and Ai *et al*., 2010). Finally, two strand displacement DNA polymerase enzymes namely *Bacillus stearothermophilus* (*Bst)* and *Bacillus smithii (Bsm)* were tested to determine which between the two had the optimum yield, while other reaction conditions being held constant. The two were chosen due to fluctuating availability in the market. From the

results of these experiments, one primer set was selected due to its ability and consistency to amplify *Ascaris* DNA in the LAMP reaction (Table 3.1).

3.11 The LAMP assay

A heat block, a water bath or a thermal cycler can be used for LAMP since incubation temperature should be constant. In this study all reactions were incubated in a thermal cycler, GeneAmp PCR System 97 (Applied BioSystems, Singapore). After several optimization reactions, the following LAMP reaction conditions were found to be suitable: 1µL of each of the four primers (FIP, BIP, B3 and F3) in a ratio of 6:1 inner to outer primers; 8 U/µL of *Bsm* DNA polymerase (Thermo Scientific); 10X *Bsm* Buffer; 1.2X of 2X LAMP reaction buffer [1M TrisHCl, KCl, MgSO4, (NH4)2SO4, Tween 20, Betaine, dNTPs] (Landis and Koch, 1997); and 1 µL target DNA in a final reaction volume of 21µL. Amplification was carried out at 63^{o}C for 45 minutes, and *Bsm* DNA polymerase activity stopped by a final incubation at 80^{o}C for 10 min. *Bsm* DNA polymerase provided better results as compared to *Bst* DNA polymerase, hence maintained for the rest of the experiment.

To detect amplicons, both gel electrophoresis and visualization using the naked eye following staining were used. For electrophoresis, 5µL of the products was separated using 2% TAE agarose gel stained with SYBR green safe DNA gel stain (Notomi *et al.*, 2000). Also, visual detection of the amplicon was carried out using SYBR Green 1 nucleic acid gel stain 10,000 (Life Technologies) *(Macuhova et al., 2010)* diluted in a 1:10 ratio, and 1µL of the diluted dye added to each amplification reaction for visual detection with the naked eye. Color change was observed and photographed using the Nikon Coolpix 4500.

3.12 Evaluation of the LAMP assay using DNA extracted directly from fecal samples in comparison with Kato-Katz

Extraction of DNA was conducted in 40 randomly selected fecal samples that were blinded for use as clinical samples from the initial after Kato-Katz results were obtained. I used the QIAamp Fast DNA stool mini kit (QIAGEN, Hilden, Germany), as per the manufacturer's instructions. Briefly, 200 mg of stool was weighed and placed into a 2 ml tube. 1 ml of InhibitEX buffer was added and vortexed for 1 min. The mixture was heat for 10 min at 95^{o}C followed by vortexing for 15 seconds. The sample was centrifuged for 1 min at 14,000rpm (full speed). In a 1.5 ml micro centrifuge tube, 15 µL of Proteinase K was added followed by addition of 200 µL of the supernatant, which was followed by a further addition of 200µl Buffer AL a lysis buffer. This mix was then vortex for 15 s. 200 µl of absolute ethanol was added and the followed by 10 sec of vortex. 600µl of the resulting lysate solution was pippeted into a spin column and centrifuged for 1 min at 14,000 rpm. The filtrate was then discarded. To the same spin column 500µl of buffer1 (AW1 a wash buffer that contains a low concentration of quinidine) was added and centrifuged for 1 min and the filtrate discarded followed by addition of 500µl of buffer 2 (AW2 a Tris-based ethanol solution, used to remove salts). This was then centrifuged for 3 minutes and the filtrate discarded. The spin column was replaced on an empty tube for another dry spin at 14,000 rpm for 3 min to remove any residual buffers. The spin column was placed onto a clean 1.5 ml tube and 200µl of DNA elution buffer added, and incubated at room temperature for 1 min. This was span for 1 min at 14,000 rpm to elute the DNA.

Samples were blinded as they had earlier been screened using Kato-Katz procedure. Briefly: Small amount of feces was compressed through a metal sieve and transferred with a wooden applicator stick to plastic templates with a 6 mm diameter hole placed on two glass slides. Once the hole was filled, the templates were removed and the stool covered with a cellophane tapes soaked in malachite green. The slides were inverted and pressed against a newspaper sheet, consequently spreading and thinning out the fecal mount. The slide was observed under a microscope at x400 magnification. A

contingency table was used to determine the percent agreement between the developed assay and Kato-Katz.

CHAPTER FOUR

RESULTS

4.1 The first internal transcribed spacer region

After comparing between mitochondrial DNA and both ITS-1/ITS-2, I found ITS-1 of ribosomal DNA to be a suitable diagnostic target marker for *Ascaris lumbricoides,* and designed primers based on the same. Its suitability as diagnostic markers was especially due to the fact that the region is conserved among species and between species, hence reproducibility of results.

4.2 Optimized conditions

4.2.1 DNA concentration and primer ratio

The worm DNA was used as a positive control for standardization of the assay. The DNA concentration of the worm was diluted to match to the lowest concentration of DNA extracted from single ovum, 10 ng/µL. The primer ratio that was amplified in the assay was 6:1 ratio of the inner to outer primer as shown on Figure 4.1

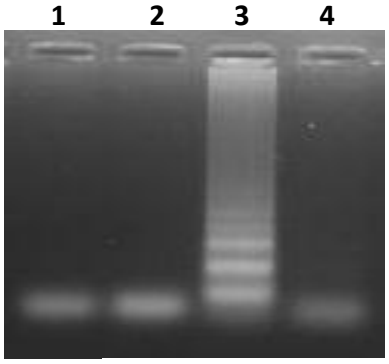

Figure 4.1: Ratio of inner to outer primers

The 2% agarose gel plate shows amplification of adult *Ascaris* worm with variation of inner to outer primer ratio. Lane 1 - 2:1, Lane 2 – 4:1, Lane 3 – 6:1, Lane 4 – 8:1. Lane 3 which had 6:1 ratio readily amplified word DNA, setting precedence for the LAMP technique.

4.2.2 Temperature

The temperature that resulted in amplification was 63 ^{o}C.

4.2.3 Time

The optimum time was found to be 45 min.

4.2.4 DNA polymerase enzyme

Bacillus smithii DNA polymerase was used in the LAMP technique as it produced results under the above mentioned conditions. One unit of *Bsm* DNA polymerase requires a temperature of 60 ^{o}C to incorporate 10 nmol of dNTPs into polynucleotide fraction in 30 min, unlike *Bst* DNA polymerase that will require a higher temperature of 65 ^{o}C for the same function at the same amount of time. *Bst* failed to produce any results under the optimized conditions.

4.3 Sensitivity of LAMP

The LAMP assay was able to amplify DNA extracted from a single egg at a DNA concentration of 10.8 ng/µL. This was confirmed by both gel electrophoresis (illustrated in Figure 2A) and with SYBR green dye (Figure 2B).

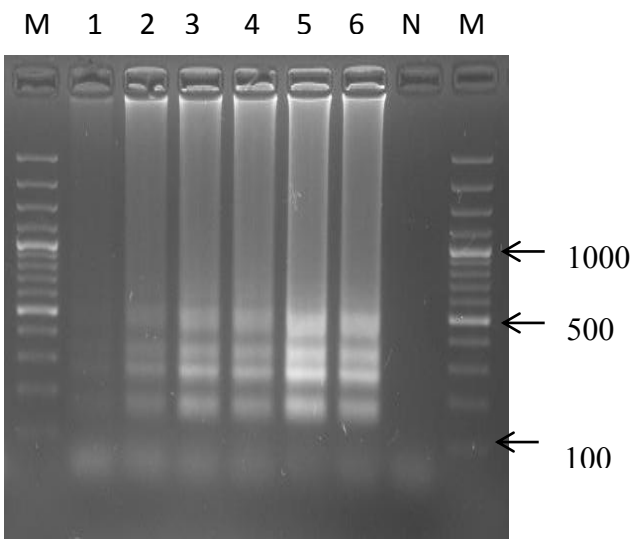

Figure 4.2 (a): Sensitivity of LAMP visualized by 2% agarose

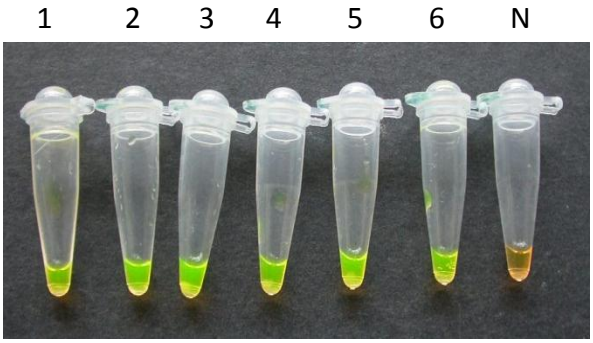

Figure 4.2 (b): Visual detection of LAMP

Fig. 4.2 (a): Lane 1, 2, 3, 4, 5, 6 shows amplified DNA extracted from 1, 5, 10, 15, 20 and 25 *A. lumbricoides* eggs respectively. Lane M is the 100bp molecular Marker and N is the negative control comprising of the LAMP master mix without DNA.

Fig. 4.2 (b): Visual detection of color change using the SYBR green dye. On addition of SYBR green dye which is orange in colour, positively amplified products turn green, and where no amplification occurred remains orange. Tubes 1, 2, 3, 4, 5, 6 represent DNA extracted from 1, 5, 10, 15, 20 and 25 *A. lumbricoides* eggs respectively through 6, Tube N - negative control comprising of the LAMP master mix without DNA.

4.4 Specificity of LAMP

When *A. lumbricoides* specific primers were used to amplify DNA from hookworm, *T. trichiura* and *S. mansoni* parasites that commonly co-occur in feces with *Ascaris*, no cross reactivity or amplification were observed as shown in Figure 5 suggesting that the LAMP reaction was specific to *A. lumbricoides*.

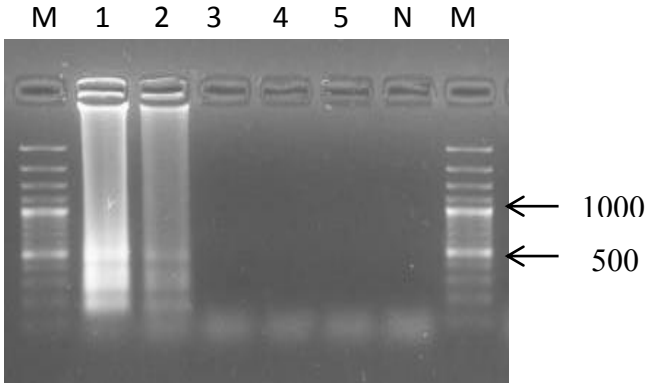

Figure 4.3 (a): Specificity of LAMP assay by gel electrophoresis.

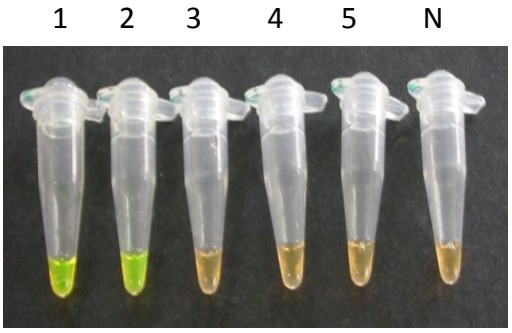

Figure 4.3 (b): Visual detection by color change using the SYBR Green Dye.

Fig. 4.3 (a): Lane 1, *A. lumbricoides* adult worm, lane 2, *Ascaris* ova DNA, lane 3, hookworm ova DNA, lane 4, *S. mansoni* ova DNA, lane 5, *T. trichiura* ova DNA; lane N, negative control and M = 100 bp molecular ruler

Fig. 4.3 (b): Tube 1 - *A. lumbricoides* adult worm, Tube 2 - *Ascaris* ova DNA, Tube 3 - hookworm ova DNA, Tube 4 - *S. mansoni* ova DNA, Tube 5 - *T. trichiura* ova DNA; Tube N - Negative control

4.5 Evaluation of the LAMP assay to Kato-Katz Technique

The developed LAMP technique was compared to Kato- Katz technique using 40 blinded samples to mimic clinical samples and results indicated in Tables 4.2 and 4.3. Both Kato-Katz and LAMP identified 26 samples as true positives and 8 as true negatives for *Ascaris* (Table 4.3). Overall, LAMP identified 31 positive samples and 9 negative samples while Kato-Katz identified less positive samples at 27, and more negative samples at 13, a clear indication that LAMP is more sensitive as compared to the gold standard.

Table 4.1: 2 x 2 contingency table comparing Kato-Katz to LAMP

		Kato-Katz (gold standard)		
		Positive	Negative	**Total**
LAMP	Positive	26	5	**31**
	Negative	1	8	**9**
Total		**27**	**13**	**40**

Sensitivity = $\frac{26}{27}$ x 100% = 96.3%

Specificity = $\frac{8}{13}$ x 100% = 61.5%

Positive predictive value = $\frac{26}{31}$ x 100% = 83.9%

Negative predictive value = $\frac{8}{9}$ x 100% = 88.9%

Table 4.2: Percent agreement of LAMP and Kato-Katz

		Kato-Katz (gold standard)		Total
		Positive	Negative	
LAMP	Positive	18.2	12.8	**31**
	Negative	8.8	0.2	**9**
		27	**13**	**40**

Percent agreement expected by chance alone = $\frac{18.2 + 0.2}{40} \times 100 = 46\%$

Kappa = $\frac{\text{(Percent agreement observed) – (Percent Agreement expected by chance alone)}}{\text{100\% - (Percent agreement expected by chance alone)}}$

$= \frac{(85 – 46)\ \%}{(100 – 46)\ \%}$

$= 0.72\ \%$

CHAPTER FIVE

DISCUSSION

Loop mediated isothermal amplification is a relatively new molecular technique (Notomo *et al*., 2000). In the study, I managed to successfully amplify DNA from both ova and worm of *Ascaris lumbricoides* by using LAMP technique. In addition, the assay was both sensitive and specific, correctly identifying true positive fecal samples with ascariaris and true negatives by failing to amplify DNA of closely related parasites that commonly co-occur with *A. lumbricoides*. Currently, there is a research gap when it comes to diagnosis of soil transmitted helminthes by LAMP, and so far *T. cati* and *T. canis* are the only nematodes to have been evaluated using LAMP (Macuhova *et al*., 2010).

The fact that LAMP could detect DNA from a single parasite ovum helps to illustrate the potential that the developed assay has for use in low infection set up characteristic of disease control/elimination situation. However, the developed LAMP technique has reduced sensitivity as compared to Kato-Katz, attributed to insufficient disruption of *Ascaris* ova which tend to have multiple proteinous layers, and presence of natural DNA polymerase inhibitors in fecal samples (Schrader *et al*, 2012). There was evidence of insufficient ova disruption by observing drops of pellets from the first step of DNA extraction using QIAamp Fast DNA stool mini kit. When a drop of pellet was placed on a slide and observed under a microscope, numerous intact *Ascaris* ova could be seen in the samples, which explained the fact that despite high EPG by Kato-Katz, there was significantly low DNA quantity of the same sample by NanoDrop.

The current efforts to eliminate ascariasis infections require simple, inexpensive, and reliable tools to detect new infections or re-infections in populations of the endemic areas, as well as to evaluate effectiveness of chemotherapy and also for epidemiological surveillance, after elimination. The developed Loop Mediated Isothermal Amplification

(LAMP) assay is suitable for use in supporting ascariasis control efforts as this study has shown that it is able to detect low amount of parasite DNA when compared with Kato-Katz. The fact that the developed assay could detect parasite DNA from a single egg suggests that even low intensity *A. lumbricoides* infections can readily be detected in this assay. It has high throughput; therefore, many samples can potentially be handled at the same time. Majority of the identified costs in the application of LAMP technique went into buying molecular reagents such as thermos-stable DNA polymerase, SYBR green DNA intercalating dye, commercially ready-to-use DNA extraction kits, and reagents for preparing LAMP buffers. A QIAmp Fast DNA stool mini kit for 50 samples was purchased at the market price of Kenya shillings (Kshs.) 33,000. This translates into Kshs. 660 for DNA extraction alone per sample. With this in mind, the estimated average cost for diagnosis of ascariasis per sample is Kshs. 800, four times higher than Kato-Katz which cost on average Kshs. 200. The modified HotSHOT which is a cheaper method of DNA extraction could only be used to extract DNA from isolated and washed ova and not directly from stool, which would require an additional purification step by use of Proteinase K.

The generated primers which were derived from the ITS-1 region of the ribosomal DNA, have been documented to be slow at accumulating substitutions and having lower intraspecific polymorphism (Egwang and Slocombe, 1982). In addition, organisms of the same genus have often been distinguished by ITS regions as they have higher sequence variability; as compared to mitochondrial DNA sequence subunits, hence the potential to differentiate between *A. lumbricoides* and *A. suum* (Fujitsu Ltd., 1999-2005). *Ascaris suum* which is a porcine nematode is morphologically indistinguishable from *A. lumbricoides.* Recent studies revealed that *A. suum* and *A. lumbricoides* differ by only six (1.3%) nucleotides in the first internal transcribed spacer (ITS-1) (Zhu *et al.*, 1999) and by 3-4% in the mitochondrial genome (mtDNA) sequence (Anderson *et al.*, 1993), indicating that the species are closely related at a phylogenetic level. The developed LAMP assay did not cross react with co-occurring helminth parasites: hookworm, *T.*

trichiura or *S. mansoni*. This is important since in many endemic localities, multiple helminth parasite infections frequently occur in an individual.

One advantage of the LAMP as an isothermal amplification method is that it eliminates heat denaturation step and use of multi-enzyme or enzyme with strand displacement to drive new round of DNA synthesis (Notomi *et al.,* 2000). Therefore sophisticated and expensive thermocycling equipment is not required for either conducting the experiment or confirming the results. Since the assay reaction is isothermal, a simple water bath can be used for this test, potentially allowing the technique to be used at the point of care in low resource disease endemic regions. This therefore means that the cost of carrying out the diagnosis is significantly reduced, making it possible for the technique to be applied in low resource setting that characterize most disease endemic areas. For instance, a simple color change detection system based on staining the amplification products with SYBR Green dye and visualized with the naked eye was possible eliminating the need for electrophoresis thus reducing cost. SYBR green is a DNA intercalating orange dye that turns green for positive results and color remains unchanged if results are negative. This also demonstrated the feasibility of using this assay under field conditions not only for simplicity. A further advantage of the LAMP technique is that during amplification, there is formation of magnesium pyrophosphate that makes the reaction mix to become turbid. This means that even the use of SYBR Green staining may not be necessary. It was also confirmed that the developed LAMP assay successfully amplified target DNA using gel electrophoresis, and the results were consistent with that of SYBR Green staining. The DNA prepared by the method described by Truent and colleagues (2000) is a simple two step procedure of lysis and neutralization and the reagents are non-toxic. Although the modified HotSHOT method lacks a DNA purification step, the specificity of LAMP was not compromised as LAMP technique was able to reliably amplify target DNA, without significant influence of the co-presence of possible non-target DNA and impurities *(*Macuhova *et al.,* 2010).

5.1 Study limitations

1. There was an overall low number of STH positive samples especially *Ascaris lumbricoides*, as most of the pupils attend both formal and Madrasa schools, most of whom had already participated in MDA exercise. Typically, ascariasis is eliminated by a single dose of 400 mg ABZ.
2. I had to incorporate a pre-DNA extraction step, which is isolation of ova from feces, which made the process of DNA extraction lengthy.
3. This was part of a larger study that aimed to simultaneously diagnose *S. mansoni* and STHs from fecal samples using LAMP, which had a specified period for completion as per the donor's instructions. The method used had to be common across all organisms of interest, which is *A. lumbricoides*, *S. mansoni*, hookworm and *T. trichiura*.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The study used the first internal transcribed spacer region of the ribosomal DNA as the suitable target for diagnosis of *A. lumbricoides* infection in fecal samples. The optimum incubation condition was found to be 63°C for 45 minutes with the ratio of inner to outer primer standing at 6:1. In comparison to the gold standard Kato-Katz microscopy based technique, LAMP was more sensitive, having detected DNA from a single *Ascaris* ovum. Therefore, LAMP could potentially be used for diagnosis of ascariasis and evaluation of effectiveness of chemotherapy-based interventions due to its simplicity and reliability.

6.2 RECOMMENDATION

1. To find a more efficient method for DNA extraction of parasite ova directly from fecal sample, to eliminate the pre-extraction step.
2. To make the developed assay more user friendly in the field set up, there is need for a pre-prepare mix that only requires addition of sample DNA.
3. To incorporate loop-primers that will significantly reduce reaction time.

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APPENDICES

Appendix I: Letter of Exemption for Pursuing Independent SSC Protocol Approval

10th July 2014

Eric Lelo, PhD
Senior Research Officer,
Centre for Biotechnology Research & Technology – KEMRI,
P.O. Box 54840-00200,
Nairobi, Kenya.
Tel: 0722759198

The Director,
Board of Postgraduate Studies.

Through,
The Principal,
College of Health Sciences.

Through,
The Program Coordinator,
ITROMID.

Dear Sir,

RE: SSC and ERC approvals for the project titled "Development of a loop-mediated isothermal amplification (LAMP) assay for diagnosis of *Ascaris lumbricoides* infection in fecal samples," conducted by Esther Andia Shiraho (TM305-2072/2012).

The student Esther Andia Shiraho of registration number TM305-2072/2012 will conduct the study titled Development and evaluation of a loop-mediated isothermal amplification (LAMP) assay for diagnosis of *Ascaris lumbricoides* infection in fecal samples. This is part of a larger on-going SSC approved protocol No. 2683 titled, ***"Development and evaluation of a LAMP technology-based test for simultaneous diagnosis of schistosomiasis and soil transmitted helminthiasis,"*** where I am the principal investigator.

The relevant approval letters from the SSC and ERC are hereby attached. Please note that the objectives addressed in the sub-project are interrelated to those of the main study, therefore be exempted from pursuing independent approvals.

Below are the objectives that will be addressed by the student:-

General Objective:

To develop a simple, reliable, and inexpensive molecular diagnosis test for detection of *Ascaris lumbricoides* infection in fecal samples.

Specific Objectives:

1. To identify a suitable DNA target for a LAMP assay for detection of *A. lumbricoides* infection in fecal samples.
2. To optimize conditions for the LAMP assay for detection of *A. lumbricoides* infection in fecal samples.
3. To evaluate the developed LAMP assay for its ability to detect *A. lumbricoides* infection in fecal samples, in comparison with microscopy-based tests and PCR.

Please accord the student any assistance necessary.

Thank you.

Yours faithfully,

Dr. Eric Lelo Agola

Appendix II: SSC Approval Letter

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

ESACIPAC/SSC/102092 **23rd September, 2013**

Eric Agola

Thro'
Director, CBRD
NAIROBI

23/9/13

REF: SSC No. 2683 (Revised) – Development and evaluation of a LAMP Technology-Based test for simultaneous diagnosis of *Schistosoma mansoni* and soil transmitted (STH) infection in fecal samples

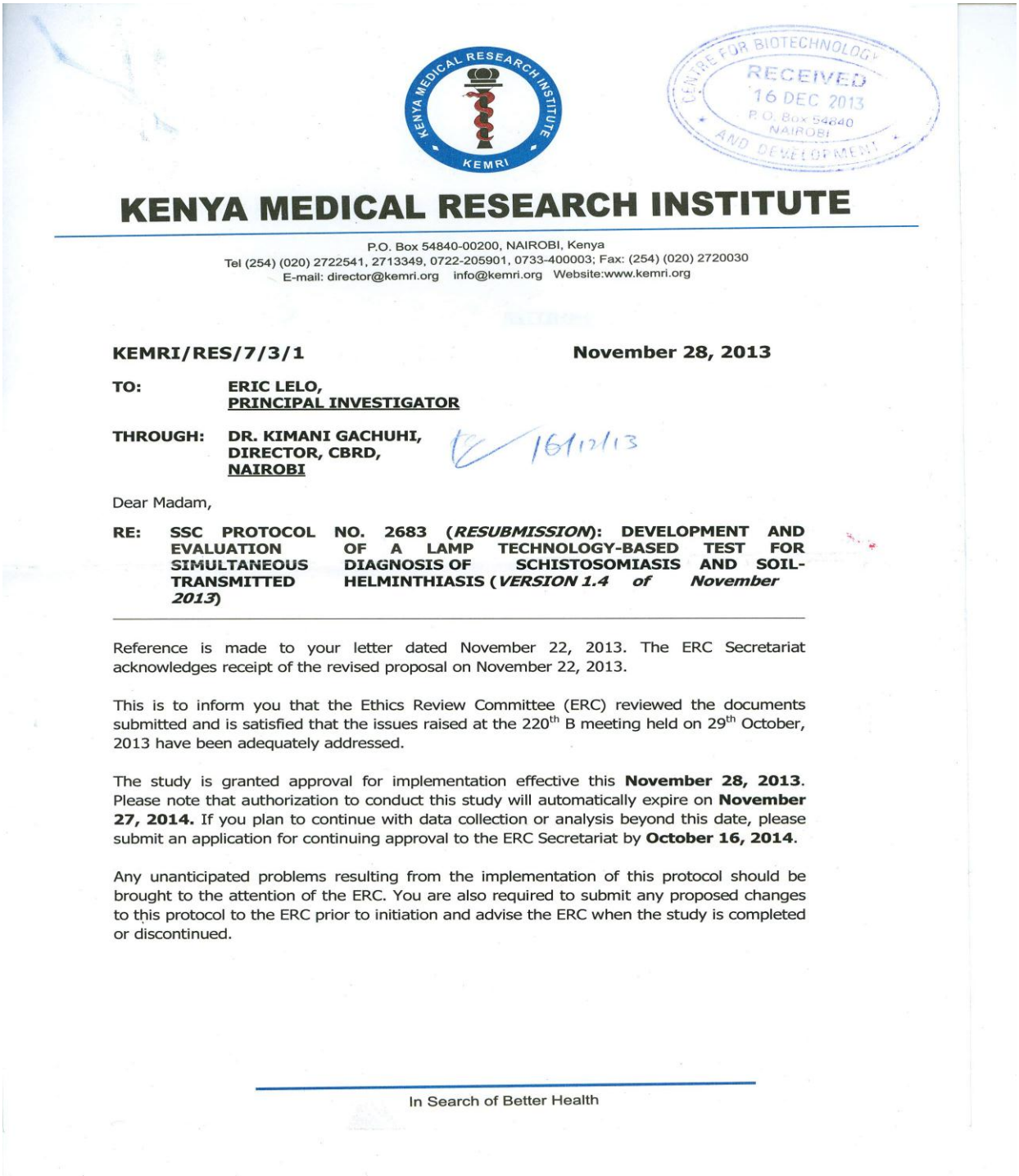
Thank you for your letter dated 19th September, 2013 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your proposal now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval.

Sammy Njenga, PhD
SECRETARY, SSC

Appendix III: ERC Approval Letter



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 **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.

This is to inform you that the Ethics Review Committee (ERC) reviewed the documents submitted and is satisfied that the issues raised at the 220th B meeting held on 29th October, 2013 have been adequately addressed.

The study is granted approval for implementation effective this **November 28, 2013**. Please note that authorization to conduct this study will automatically expire on **November 27, 2014.** If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **October 16, 2014**.

Any unanticipated problems resulting 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

You may embark on the study.

Yours faithfully,

EAB

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

Appendix IV: Informed Consent Agreement for Parents/Guardians

Informed Consent Agreement for Parents/Guardians

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 V: Assent Form

Assent for Children Aged 13 -15:

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 also, 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. Actually, 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 VI: Publication