

**DEVELOPMENT AND EVALUATION OF A MODIFIED
LOOP-MEDIATED ISOTHERMAL AMPLIFICATION
(mLAMP) TEST FOR THE DETECTION OF
*ENTAMOEBIA HISTOLYTICA***

FRIDAH MWENDWA KIRIMI

MASTER OF SCIENCE

(Molecular Medicine)

**JOMO KENYATTA UNIVERSITY OF
AGRICULTURE AND TECHNOLOGY**

2018

**Development and Evaluation of a Modified Loop-Mediated Isothermal
Amplification (mLAMP) Test for the Detection of *Entamoeba*
*histolytica***

Fridah Mwendwa Kiriimi

**A thesis submitted in partial fulfillment of the requirements for the
degree of Master of Science in Molecular Medicine in the Jomo
Kenyatta University of Agriculture and Technology**

2018

DECLARATION

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

Signature Date

Fridah Mwendwa Kirimi

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

Signature Date.....

Dr. Johnson Kinyua, PhD
JKUAT, Kenya

Signature Date.....

Dr. Cecilia Kathure Mbae, PhD
KEMRI, Kenya

Signature Date.....

Prof. Gitonga Nkanata Mburugu, PhD
Meru University of Science and Technology, Kenya

Signature Date.....



Dr. Zablon Njiru, PhD
Murdoch University, Australia

DEDICATION

I dedicate this thesis to my family, The Kirmis.

ACKNOWLEDGEMENTS

I would like to acknowledge my supervisors Dr. Johnson Kinyua, Dr. Cecilia Mbae, Prof. Gitonga N. Mburugu and Dr. Zablon Njiru for their expert guidance in the design, execution and report write-up of this work.

Staff members of Centre for Microbiology Research, Parasitology lab, KEMRI, ESACIPAC laboratory and Aga Khan University Hospital Pathology department I thank you for your support while I used your facilities to carry out this research work. Special thanks to Dr. Eberhard Zeyhle.

I acknowledge financial support for the research work from National Commission for Science, Technology and Innovation, NACOSTI, Kenya, www.nacosti.go.ke. Special gratitude to Meru University of Science and Technology for the provision of extra materials.

Further, I acknowledge my parents for moral and financial support towards the undertaking of this Master's program. Special recognition to my mum, Salome, for her relentless encouragement, support and prayers. I also thank my siblings for their support.

Finally yet importantly, I thank God for enabling me do it all.

TABLE OF CONTENTS

DECLARATION	ii
DEDICATION	iii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	v
LIST OF TABLES	ix
LIST OF FIGURES	x
LIST OF APPENDICES	xi
ABBREVIATIONS AND ACRONYMS	xii
ABSTRACT	xv
CHAPTER ONE	1
INTRODUCTION	1
1.1 Background Information	1
1.2 Statement of the Problem	4
1.3 Justification of the study.....	5
1.4 Objectives	6
1.4.1 General objective	6
1.4.2 Specific objectives	6
1.5 Significance of the study	6

1.6	Limitation of the study	6
CHAPTER TWO.....		7
LITERATURE REVIEW.....		7
2.1	Epidemiology of amoebiasis	7
2.2	Pathophysiology of <i>Entamoeba histolytica</i>	8
2.3	Diagnosis of <i>Entamoeba histolytica</i>	9
2.3.1	Microscopy.....	9
2.3.2	Culture and isoenzyme analysis	11
2.3.3	Antibody tests.....	13
2.3.4	Antigen tests	13
2.3.5	Polymerase Chain Reaction, PCR.....	14
2.3.6	Loop-mediated isothermal amplification, LAMP	15
2.4	<i>Entamoeba histolytica</i> genome.....	19
2.5	Hemolysin (HLY6) gene	20
2.6	18S rRNA gene.....	20
CHAPTER THREE		21
MATERIALS AND METHODS.....		21
3.1	Study design and location.....	21
3.2	Sample size determination.....	21

3.3	Clinical samples	22
3.4	DNA Extraction.....	23
3.5	Cultivation of <i>E. histolytica</i>	23
3.6	Polymerase Chain Reaction test.....	24
3.7	Design of LAMP primers	25
3.8	LAMP reactions	26
3.9	Analytical sensitivity LAMP test	27
3.10	Detection and confirmation of LAMP product	28
3.11	Data management and analysis	28
3.12	Reference DNA	29
3.13	Ethical clearance.....	30
CHAPTER FOUR.....		31
RESULTS		31
4.1	LAMP primers.....	31
4.2	<i>Entamoeba histolytica</i> LAMP optimum reaction conditions	33
4.3	Analytical sensitivity of LAMP and PCR tests	34
4.4	<i>Entamoeba histolytica</i> LAMP product.....	38
4.5	Results for clinical samples	41

CHAPTER FIVE	43
DISCUSSION, CONCLUSION AND RECOMMENDATION	43
5.1 Discussion.....	43
5.2 Conclusion	47
5.3 Recommendation	47
REFERENCES	48
APPENDICES	64

LIST OF TABLES

Table 4.1: Nucleotide sequences for <i>E. histolytica</i> primers for stem 18S rRNA and HLY6 LAMP tests.	32
Table 4.2: The analytical sensitivity of 18S rRNA LAMP test combinations and PCR test using a 10-fold serial dilution of <i>E. histolytica</i> DNA	35
Table 4.3: Comparative evaluation of Stem-18S LAMP, Standard LAMP and PCR tests in detection of <i>E. histolytica</i> DNA in clinical samples (n = 126).	41
Table 4.4: Comparative analysis of the molecular-based methods in the detection of <i>E. histolytica</i> DNA in clinical samples using Kappa Coefficient.	42

LIST OF FIGURES

Figure 2.1: Diagnostic and infective stages of <i>Entamoeba histolytica</i> (CDC, 2009)	11
Figure 2.2: The LAMP principle. Starting material producing step (1-5), Cycling amplification step (5-8) and Elongation step (9-12).....	18
Figure 4.1: Optimization of stem 18S <i>E. histolytica</i> LAMP test reactions.....	33
Figure 4.2: Sensitivity and specificity analysis.....	36
Figure 4.3: Sensitivity of PCR test.....	37
Figure 4.4: Specificity of the stem 18S LAMP assay.....	37
Figure 4.5: (a) The real time curves acquired using <i>E. histolytica</i> stem 18S-LAMP test as monitored using the real time PCR machine.....	39
Figure 4.6: Ddei restriction enzyme digestion of stem 18S LAMP test product.....	40

LIST OF APPENDICES

Appendix 1: Ethical approval	64
Appendix 2: Consent forms	65
Appendix 3: Protocol - Isolation of DNA from Stool for Pathogen Detection.....	79
Appendix 4: Protocol for stem 18S LAMP test for <i>Entamoeba histolytica</i>	83
Appendix 5: Publication	85

ABBREVIATIONS AND ACRONYMS

AIDS	Acquired immune deficiency syndrome
ASL buffer	Stool lysis buffer
B3	Backward outer primer
BIP	Backward inner primer
BLAST	Basic local alignment search tool
bp	Base pair
<i>Bst</i>	<i>Bacillus stearothermophilus</i>
C_T	Cycle threshold
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent assay
F3	Forward outer primer
FAM	Carboxyfluorescein
FIP	Forward inner primer
g	Gram
GAL	Galactose
GalNAc	<i>N</i> -acetylgalactosamine
HCl	Hydrochloride
HIV	Human immunodeficiency virus
HLY6	Hemolysin 6

ID	Identification
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KCl	Potassium chloride
KEMRI	Kenya Medical Research Institute
Kbps	Kilo base pairs
LAMP	Loop-mediated Isothermal Amplification
LB	Loop backward primer
lbs	Pounds-mass
LF	Loop forward primer
Mbps	Mega base pairs
MgCl₂	Magnesium chloride
MgSO₄	Magnesium sulphate
ml	Milliliter
mm	Millimeter
mM	Millimolar
NTD	Neglected Tropical Disease
NACOSTI	National Commission for Science, Technology and Innovation
PAHO	Pan American Health Organization
PCR	Polymerase chain reaction
pH	Power of hydrogen
Pmol	Picomole

qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
SB	Stem backward primer
SF	Stem forward primer
SSC	Scientific Steering Committee
<i>Taq</i>	<i>Thermus aquaticus</i>
T_m	Melting temperature
U	Unit
UK	United Kingdom
UNESCO	United Nations Educational, Scientific and Cultural Organization
USA	United States of America
UV	Ultra violet
UVP	Ultra Violet Products
V	Volts
WHO	World Health Organization
(NH₄)₂SO₄	Ammonium sulphate
°C	Degree celsius
μl	Microliter
μM	Micromolar
%	Percentage

ABSTRACT

Entamoeba histolytica, the causative agent for amoebiasis is of considerable burden to populations in the developing countries where it accounts for over 50 million infections. The tools for detection of amoebiasis are inadequate and diagnosis relies on microscopy which means a significant percent of cases remain undiagnosed. Moreover, tests formats that can be rapidly applied in rural endemic areas are not available. This study aimed at developing a rapid modified LAMP test that can be applied in the detection of *E. histolytica*. Three hundred and thirty four samples were collected and microscopically examined for the presence of *E. histolytica*, from children who presented with diarrhea, abdominal pain and/or discomfort at three participating outpatient clinics at Mukuru informal settlements and one inpatient paediatric ward of Mbagathi District Hospital. Microscopic examination scored 126 of the 334 samples as positive for *Entamoeba*. A loop-mediated isothermal test (LAMP) based on 18S small subunit ribosomal RNA gene was designed with a pair of extra reaction accelerating primers (stem primers) and compared with the published LAMP and PCR tests in detection of *Entamoeba histolytica* DNA in clinical samples. The stem LAMP test indicated shorter time to results by an average 11 min and analytical sensitivity of 10^{-7} (~30 pg/ml) compared to the standard LAMP and PCR which showed sensitivities levels of 10^{-5} (~3 ng/ml) and 10^{-4} (~30 ng/ml) respectively using ten-fold serial dilution of DNA. In the analysis of microscopy *Entamoeba* spp trophozoites and cysts positive clinical samples, stem LAMP detected *Entamoeba histolytica* DNA in 36/126, standard LAMP test 20/126 and PCR 18/126 respectively. There was 100% agreement in detection of the stem LAMP product using fluorescence of SYTO-9 dye in real time machine, through addition of 1/10 dilution of SYBR® Green I and electrophoresis in 2% agarose gel stained with ethidium bromide. A new stem 18S LAMP test which is a modification of the standard LAMP test through inclusion of stem primers was developed. It is recommended that this new stem 18S LAMP test be further evaluated using a larger sample size and be part of diagnostic algorithms for amoebiasis.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Amoebiasis, caused by a protozoan *Entamoeba histolytica* is an important human gastrointestinal infection responsible for over 100, 000, 000 amoebic infections each year (WHO, 2015). Most of these cases are recorded in the developing countries (Mbae *et al.*, 2013; Samie *et al.*, 2006; Gatei *et al.*, 2006). In Africa, World Health Organization estimated 796 per 100,000 median rate of *E. histolytica* infections in 2010 (WHO, 2015). Extrapolating these results, this becomes a considerable burden to populations in countries with low economies and plagued with other diseases such as malaria, HIV-AIDS, tuberculosis and non-communicable diseases.

The appropriate management of amoebiasis is dependent on accurate diagnosis, however, the available diagnostic tools are inadequate. The mostly used conventional method is microscopy. However, microscopy has several important disadvantages such as low sensitivity of less than 60% and it fails to differentiate *E. histolytica* from the morphologically similar non-pathogenic species.

The culturing of stool followed by isoenzyme analysis has been used and enable species differentiation (Huston *et al.*, 1999) however these methods are time consuming, require

special laboratory facilities hence impractical for use in the routine diagnosis of intestinal amebiasis.

Enzyme-linked Immunosorbent assay (ELISA) tests detecting *E. histolytica* antigens in stool have been developed (Gonin & Trudel, 2003; Evangelopoulos *et al.*, 2001; Abd-Alla *et al.*, 2000) and indicate sensitivity of 86-95% compared to microscopy/culture (Haque *et al.*, 1997) and real-time PCR (Roy *et al.*, 2005). The limitation of this ELISA is cross-reactivity with *E. dispar* (Visser *et al.*, 2006; Furrows *et al.*, 2004). There are no specific antigen tests available for the detection of *E. dispar* and *E. moshkovskii* from clinical samples (Fotedar *et al.*, 2007a).

Various DNA amplifying tests are available for detection and differentiation of *E. histolytica* from non-pathogenic *E. dispar* and *E. moshkovskii* (Khairnar & Parija, 2007; Freitas *et al.*, 2004; Pinheiro *et al.*, 2004; Gonin & Trudel, 2003). One such method is a novel nested multiplex PCR based on 16S-like rRNA gene that can detect and differentiate all the three morphologically similar forms of *E. histolytica*, *E. moshkovskii* and *E. dispar* simultaneously in stool samples (Khairnar & Parija, 2007). These PCR based tests have showed high specificity and sensitivity, but despite the reported advantage, the method has limited use in routine diagnosis of amoebiasis in Kenya due to associated cost.

So far there is no single reliable test that can be used in detection of amoebiasis nor be applicable under field conditions in the endemic areas. This implies a significant percent of cases go undiagnosed and continues to be a source of infection within the population.

In the last decade, a rapid DNA amplification test called loop-mediated isothermal amplification (LAMP) of DNA (Notomi *et al.*, 2000) has been developed. The LAMP technique is a potential point of care platform.

The LAMP technique has recently been applied in detection of human diseases such as malaria (Chahar *et al.*, 2017; Tegegne *et al.*, 2017) human toxoplasmosis (Sun *et al.*, 2017) and meningitis (Lee *et al.*, 2016) and has been hypothesized to revolutionize field based molecular test (Notomi *et al.*, 2015; Lee, 2017). In addition, LAMP has recently been used successfully to detect other human stool pathogens such as *Ascaris lumbricoides* (Shiraho *et al.*, 2016), *Clostridium difficile* (Wei *et al.*, 2015) and hookworms (Mugambi *et al.*, 2015).

Previously, the LAMP tests for *E. histolytica* have been developed based on small subunit (ss) of ribosomal gene (Liang *et al.*, 2009) and HLY6 gene (Rivera & Ong, 2013). However, both LAMP tests have showed lower sensitivities to respective PCR test targeting the same sequences respectively. In that context, the ssLAMP indicated sensitivity of 1 parasite/ml compared to the nested PCR which detected 0.1 parasite/ml (Liang *et al.*, 2009) while the HLY6 based LAMP test detected 5 parasites per reaction (15.8 ng/ μ L of DNA) compared to PCR with 2 ng/ μ L DNA (Rivera & Ong, 2013). Thus, these LAMP tests cannot be reliably used in detection of *E. histolytica*.

Since LAMP method presents a unique technique that may be applicable under the field conditions to diagnose *E. histolytica* in endemic areas, it is sensible to continue modifying the technique to improve the sensitivity levels. To achieve this, this study

needed to develop a new LAMP test based on multi-copy (200 copies/cell) small-subunit ribosomal DNA in the *E. histolytica* genome (Liang *et al.*, 2009). The LAMP test sensitivity level was set to improve by designing a novel second set of reaction accelerating primers that work with loop primers (Notomi *et al.*, 2000).

1.2 Statement of the Problem

The tests available for diagnosis of amebiasis such as microscopy (the gold standard) and stool culture are inadequate due to their low sensitivity (less than 60%), inability to distinguish pathogenic from non-pathogenic species and long turnaround time (Gonzalez-Ruiz *et al.*, 1994). This means a significant number of infected cases remain undiagnosed and there are unnecessary treatments that may lead to rise of drug resistance. Consequently, development of definitive test(s) is a priority.

LAMP tests for *E. histolytica* have been developed. However, these tests have shown low sensitivity thus PCR test targeting the same sequence show higher sensitivity. The ssLAMP indicated sensitivity of 1 parasite/ml compared to the nested PCR which detected 0.1 parasite/ml (Liang *et al.*, 2009) while the HLY6 based LAMP test detected 5 parasites per reaction (15.8 ng/ μ L of DNA) compared to PCR with 2 ng/ μ L DNA (Rivera & Ong, 2013). Therefore they cannot be reliably used in detection of *E. histolytica*. This study sought to develop an improved LAMP test based on *E. histolytica* small-subunit ribosomal DNA that which would improve sensitivity and reduce reaction time.

1.3 Justification of the study

Amoebiasis, caused by *E. histolytica* affects over 100 million people worldwide. The highest prevalence of *Entamoeba* infections is found in countries in subtropical and tropical regions with low hygienic standards, and high population density (Tannich & Burchard, 2010; WHO/PAHO/UNESCO report, 1997; Walsh, 1989). In Kenya the true prevalence of amoebiasis cases is not known. However, prevalence of 7% and 12-32% has been recorded in children and adult respectively using microscopy (Saidi *et al.*, 1997; Nyarango *et al.*, 2008; Kagira *et al.*, 2011). This is a considerable burden to populations and countries with low economies and plagued with other diseases such as malaria, HIV-AIDS, tuberculosis and non-communicable diseases.

Mukuru informal settlement was chosen as study location as it has high population density and low hygienic standards, which are prerequisites for high prevalence of amoebiasis (Tannich & Burchard, 2010). The LAMP technology shows potential of improving disease diagnostic landscape and in particular the development of point of care tools. The technology is simple to perform, rapid, does not require sophisticated equipment, is easily adaptable for visual detection of results and can be performed under field conditions in resource-limited countries (Njiru *et al.*, 2008a). LAMP has been used to diagnose other human stool pathogens such as *Ascaris lumbricoides* (Shiraho *et al.*, 2016), *Clostridium difficile* (Wei *et al.*, 2015) and hookworms (Mugambi *et al.*, 2015). Modified LAMP with stem primers has benefited diagnosis for diseases such as *Clostridium difficile*, *Listeria monocytogenes* and HIV (Gandelman *et al.*, 2011). Therefore, the target for this project was to develop a sensitive *E. histolytica* LAMP test

based on the readily available multi-copy small sub-unit ribosomal gene that contributes towards diagnosis of amoebiasis.

1.4 Objectives

1.4.1 General objective

To develop and evaluate a modified loop-mediated isothermal amplification test for the detection of *Entamoeba histolytica* in Kenya

1.4.2 Specific objectives

1. To develop an 18S stem LAMP test for *E. histolytica*
2. To evaluate the 18S stem LAMP test against the published PCR and LAMP tests based on small subunit of the ribosomal and HLY6 gene targets

1.5 Significance of the study

Following the lack of a reliable test that can be used to diagnose amoebiasis, this study developed and evaluated a modified LAMP test that has potential for use as a point of care diagnostic tool.

1.6 Limitation of the study

This study did not have the financial capability to sequence test evaluation samples.

CHAPTER TWO

LITERATURE REVIEW

2.1 Epidemiology of amoebiasis

Amoebiasis, caused by protozoan *Entamoeba histolytica* is an important human gastrointestinal infection worldwide. The World Health Organization (WHO) estimates that over 100 million people worldwide suffer from invasive amoebic infection each year (WHO, 2015). Most of these cases are recorded in the developing countries of central and South America, Africa, and the Indian sub-continent (Samie *et al.*, 2006). In industrialized countries, the disease occurs more commonly in sexually active homosexual men, immigrants and tourists who have travelled from endemic areas (Hal *et al.*, 2007; Herbinger *et al.*, 2011).

In Africa, data on true prevalence and incidence of *E. histolytica* is largely lacking due to lack of adequate funding, systematic reporting and lack of definitive tools that can unequivocally differentiate *E. histolytica* and *E. dispar* (Stauffer *et al.*, 2006; Gonin & Trudel, 2003).

Most studies indicate that the *E. histolytica* and *E. dispar* occur together. On this context, studies conducted in Kenya indicate prevalence of 6% of *E. histolytica/E. dispar* from selected hospitals (Gatei *et al.*, 2006), 7.8% in children under 5 years attending Malindi hospital (Saidi *et al.*, 1997) and 10.7% in children living in Mukuru slum areas of Nairobi (Mbae *et al.*, 2013). In adults, higher incidences of 11.9% have

been reported among the food handlers in Kisii municipality (Nyarango *et al.*, 2008) and 32.3% prevalence in patients attending Alupe hospital in Busia (Kagira *et al.*, 2011). Noting that all this data was obtained using microscopy method and its sensitivity is reported to be less than 60%, then the prevalence of *E. histolytica*/*E. dispar* is expected to be much higher than reported. A study carried out at a county referral hospital in Kenya to differentiate *E. histolytica* and *E. dispar* complex by multiplex PCR reported 16.7% samples positive for *E. histolytica* and 75% samples for *E. dispar* (Gachuhi *et al.*, 2014). The prevalence of *E. histolytica* by qPCR was recorded at 15% in Bungoma County, Western Kenya (Easton *et al.*, 2016) while a much lower prevalence of 0.4% was reported among children with vertically transmitted HIV infection (Matey *et al.*, 2016).

2.2 Pathophysiology of *Entamoeba histolytica*

Non-human primates and humans are the only natural hosts of *E. histolytica*. The *E. histolytica* infection is capable of causing several intestinal conditions including acute proctocolitis, toxic megacolon, and chronic non-dysenteric colitis and in some instances the disease may mimic acute appendicitis (Andreade *et al.*, 2007). Extra-intestinal conditions such as liver abscess, peritonitis and genitourinary disease have also been indicated with liver abscess being the most common (Rao *et al.*, 2009).

Ingestion of *E. histolytica* cysts from the environment is followed by excystation in the terminal ileum or colon to form highly motile trophozoites. Upon colonization of the colonic mucosa, the trophozoite may encyst and is then excreted in the feces, or it may

invade the intestinal mucosal barrier and gain access to the bloodstream, whereby it is disseminated to the liver, lung, and other sites. Excreted cysts reach the environment to complete the cycle.

Only a small number of cysts may cause disease. The adherence of trophozoites to colonic epithelial cells seems to be mediated by a galactose/*N*-acetylgalactosamine (GAL/GalNAc)-specific lectin, a 260-kd surface protein containing a 170-kd subunit and a 35-kd subunit (Stanley, 2003; Ximénez *et al.*, 2010). A mucosal immunoglobulin A (IgA) response against this lectin can result in fewer recurrent infections (Haque *et al.*, 2006). Spread of amebiasis to the liver occurs via the portal blood. The pathogenic strains evade the complement-mediated lysis in the bloodstream. Trophozoites that reach the liver create unique abscesses with well-circumscribed regions of dead hepatocytes surrounded by few inflammatory cells and trophozoites and unaffected hepatocytes. These findings suggest that *E. histolytica* organisms are able to kill hepatocytes without direct contact (Stanley, 2003).

2.3 Diagnosis of *Entamoeba histolytica*

2.3.1 Microscopy

The conventional method for diagnosis of *E. histolytica* utilizes microscopy. Trophozoites and cysts of *E. histolytica*, *E. dispar* and *E. moshkovskii* are identified first by light microscopic examination of fecal specimen through direct, concentrated or permanently stained smears (Figure 2.1). Motile trophozoites are examined from freshly

obtained stool, while cysts can be observed after the stool has been stored in appropriate fixatives (Fotedar *et al.*, 2007a; Pritt & Clark, 2008).

This method has a number of limitations: (i) correct identification depends greatly on the experience and skills of the microscopist- false-positive results due to misidentification of macrophages as trophozoites, PMNs as cysts (especially when lobed nuclei of PMNs break (Tanyuksel *et al.*, 2003; Gonzalez-Ruiz *et al.*, 1994); (ii) sensitivity is less than 60% even under optimal standards (Gonzalez-Ruiz *et al.*, 1994). (iii) microscopic examination of stool fails to differentiate *E. histolytica* from the morphologically similar non-pathogenic species *E. dispar* and amphizoic *E. moshkovskii* (WHO/PAHO/UNESCO report, 1997); and (iv) in settings with relatively large numbers of negative results, microscopy can be tedious, with relatively high costs for each case detected.

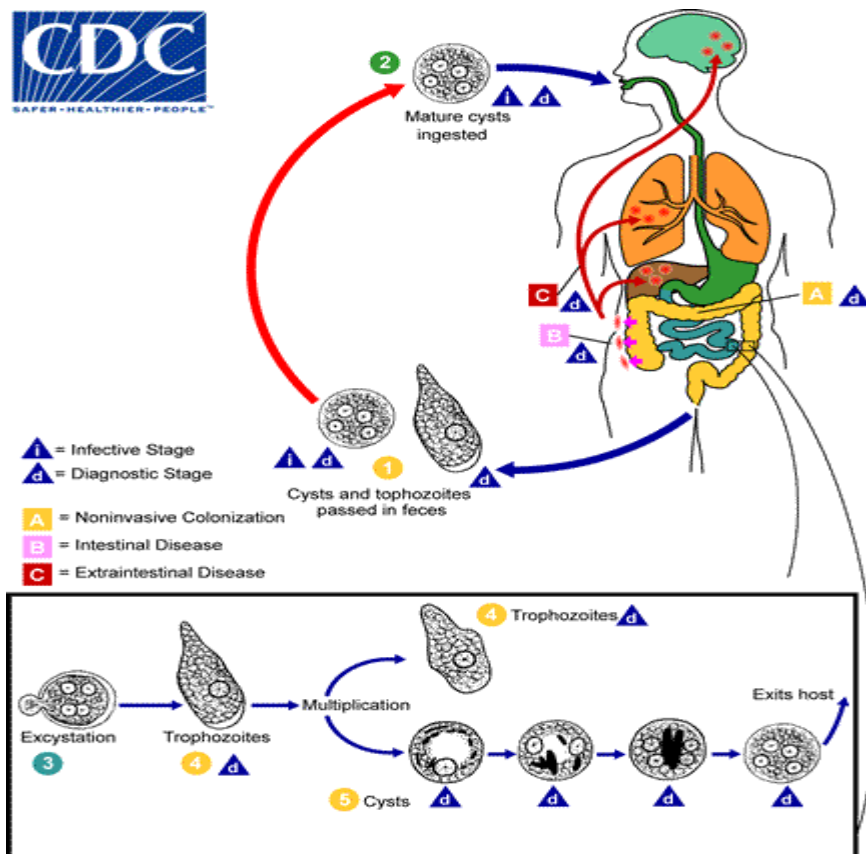


Figure 2.1: Diagnostic and infective stages of *Entamoeba histolytica* (CDC, 2009)

2.3.2 Culture and isoenzyme analysis

Culture of *E. histolytica* is performed under xenic (diphasic and monophasic) and axenic systems. Xenic cultivation is defined as the growth of the parasite in the presence of an undefined flora (Clark & Diamond, 2002). Axenic cultivation involves the cultivation of parasites in the absence of any other metabolizing cells (Clark & Diamond, 2002). Currently TYI-S-33 (Diamond *et al.*, 1978) and YI-S (Diamond *et al.*, 1995) are the most widely used media for axenic cultivation of *E. histolytica* (Clark & Diamond, 2002).

Culture of *E. histolytica* can be performed from fecal specimens, rectal biopsy specimens, or liver abscess aspirates. As the liver abscess aspirates of ALA patients are usually sterile (98% cases) (Blessman *et al.*, 2002), addition of a bacterium or a trypanosomatid is necessary before inoculation of amebae into xenic culture (Clark & Diamond, 2002; Wang *et al.*, 1973).

The success rate for culture of *E. histolytica* is between 50 and 70% in reference laboratories (Clark & Diamond, 2002). As culture of *E. histolytica* from clinical samples such as feces or liver abscesses has a significant false-negative rate and is technically difficult, it is not undertaken in a routine clinical laboratory.

Isoenzyme (zymodeme) analysis of cultured amebae enables differentiation of *E. histolytica* from *E. dispar* and was considered the gold standard for diagnosing amebic infection prior to development of newer DNA-based techniques. (Fotedar *et al.*, 2007a) Zymodemes consist of electrophoretic patterns of malic enzyme, hexokinase, glucose phosphate isomerase, and phosphoglucomutase isoenzyme (Sargeant *et al.*, 1987). Zymodeme analysis has a number of disadvantages, including the difficulty of performing the test. It is a time-consuming procedure and relies on establishing the amebae in culture, with a large number of cells needed for the enzyme analysis. This process is not always successful. The cultivation of amebae may lead to selection bias, and one species or strain may outgrow the other, which is not desirable when studying zymodemes. Furthermore, the amebic cultures and therefore isoenzyme analyses are negative for many microscopy-positive stool samples (Gonzalez-Ruiz *et al.*, 1994; Haque *et al.*, 1995). Zymodeme analysis is not easily incorporated into routine clinical

laboratory work because of the expertise required to culture the parasites, the complexity of the diagnostic process, and the cost (Fotedar *et al.*, 2007a).

2.3.3 Antibody tests

Serologic tests detect the presence of species-specific antibodies in patient's serum. The disadvantage of serologic tests is that they cannot distinguish between past and current infections. Immunoglobulin M antibodies are short-lived and can be detected during the present or current infections. In contrast, IgG antibodies are long-lived and highly prevalent in endemic areas because of past exposure (Fotedar *et al.*, 2007a; Pritt & Clark, 2008). Detection of antibodies can be helpful in the case of ALA where patients do not have detectable parasites in feces (Fotedar *et al.*, 2007a).

2.3.4 Antigen tests

Fecal antigen detection tests use specific monoclonal or polyclonal antibodies to detect *E. histolytica* antigens. TechLab *E. histolytica* II kit (TechLab, Blacksburg, VA) can detect *E. histolytica* antigens in stool specimen. This kit has shown high sensitivity 86-95% compared to microscopy, culture (Haque *et al.*, 1995; 1997; 1998) and real-time PCR (Roy *et al.*, 2005). The limitation of this ELISA kit is cross-reactivity, since samples positive for *E. dispar* may give false-positive results (Evangelopoulos *et al.*, 2001; Visser *et al.*, 2006). This test also suffers from the disadvantage that the antigens detected are denatured by fixation of the stool sample, therefore limiting testing to fresh or frozen samples.

2.3.5 Polymerase Chain Reaction, PCR

Polymerase Chain Reaction methods such as conventional, nested, multiplex and real-time have been developed for detection and differentiation of *E. histolytica* from non-pathogenic *E. dispar* and *E. moshkovskii* (Rivera *et al.*, 1996; Mirelman *et al.*, 1997; Evangelopoulos *et al.*, 2000; Verweij *et al.*, 2000; Nuñez *et al.*, 2001; Gonin & Trudel, 2003; Freitas *et al.*, 2004; Pinheiro *et al.*, 2004; Khairnar & Parija, 2007).

The successful use of PCR in studying the epidemiology of *Entamoeba* infection was first reported by Acuña-Soto *et al.* (1993). Those authors used DNA extracted directly from feces, avoiding the need to culture trophozoites, and the primers were targeted to amplify the extrachromosomal circular DNA.

Real-time PCR is a new and a very attractive methodology for laboratory diagnosis of infectious diseases because of its characteristics that eliminate post-PCR analysis, leading to shorter turnaround times, a reduction in the risk of amplicon contamination of laboratory environments, and reduced reagent costs (Klein, 2002). This approach allows specific detection of the amplicon by binding to one or two fluorescence-labeled probes during PCR, thereby enabling continuous monitoring of amplicon (PCR product) formation throughout the reaction (Fotedar *et al.*, 2007a).

2.3.6 Loop-mediated isothermal amplification, LAMP

Loop-mediated isothermal amplification (LAMP) of DNA is a novel strategy for gene amplification, which relies on strand displacement synthesis of DNA using *Bst* DNA polymerase and under isothermal conditions (60-65°C). *Bst* polymerase, which is from *Bacillus stearothermophilus*, can synthesize a new strand of DNA while simultaneously displacing the complementary strand thereby enabling DNA amplification at a single temperature with a single enzyme (Aonuma *et al.*, 2009).

LAMP has several advantages namely; (i) high sensitivity with detection levels equal or higher to those of respective PCR targeting the same gene, (ii) does not require expensive thermal cycler because amplification can be performed at a constant temperature using a normal water bath or a heating block, (iii) the amplification specificity is high since LAMP reaction uses four to six specially designed primers that recognize six to eight distinct regions on the target DNA sequence (Nagamine *et al.*, 2002), (iv) the reactions are rapid and give results in less than 1 hour (Notomi *et al.*, 2000), (v) the technique can amplify pathogen DNA from partially processed or non-processed samples, hence DNA extraction is not necessary (Poon *et al.*, 2006; Njiru *et al.*, 2008a) and (vi) one step amplification (Liang *et al.*, 2009) (vii) higher yield (Notomi *et al.*, 2000) and (viii) the products formed allow the use of different visual detection formats (Mori *et al.*, 2001; Poon *et al.*, 2006).

Since LAMP presents a unique method that can be utilized to better the diagnostic landscape of various pathogens, it is prudent to continue modifying the method for even better results. On this context, Gandelman et al. (2011) designed and applied stem primers (called so because they target the stem section of the LAMP amplicon) to improve amplification speed and reproducibility of *Clostridium difficile*, *Listeria monocytogenes* and HIV LAMP tests. Recently Njiru et al. (2017) used stem primers to increase the sensitivity of *Trypanosoma brucei gambiense* LAMP test by 10-100 fold compared to the standard LAMP test. The advantage of stem primers is that they can either be used in multiplex with loop primers (Nagamine *et al.*, 2002) or as a replacement of loop primers (Gandelman *et al.*, 2011) without affecting the test sensitivity or reproducibility.

LAMP uses many reaction components, which is a major cost in the developing countries (Njiru, 2012). However, several companies have come up with ready to use commercial isothermal master mixes slightly reducing the cost burden and need for protracted optimization procedures. These include Optigene, UK (<http://www.optigene.co.uk/products-reagents/>) and EIKEN Chemical Co Ltd, Japan (<http://www.eiken.co.jp/en/>).

2.3.6.1 Principle of LAMP

LAMP amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. An

inner primer containing sequences on the sense and anti-sense 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 cycling reaction continues with accumulation of 10^9 copies of 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 target in the same strand (Notomi *et al.*, 2000; Figure 2.2).

Although originally designed to utilize 4 primers (Notomi *et al.*, 2000), optimal performance of the isothermal amplification (LAMP) requires a total of 6 primers (Nagamine *et al.*, 2002): 2 loop generating primers, 2 displacement primers, and 2 “Loop primers” (Loop B and Loop F). The method relies on auto cycling strand displacement DNA synthesis performed by DNA polymerase with high strand displacement activity, and a set of two specifically designed inner primers and two outer primers.

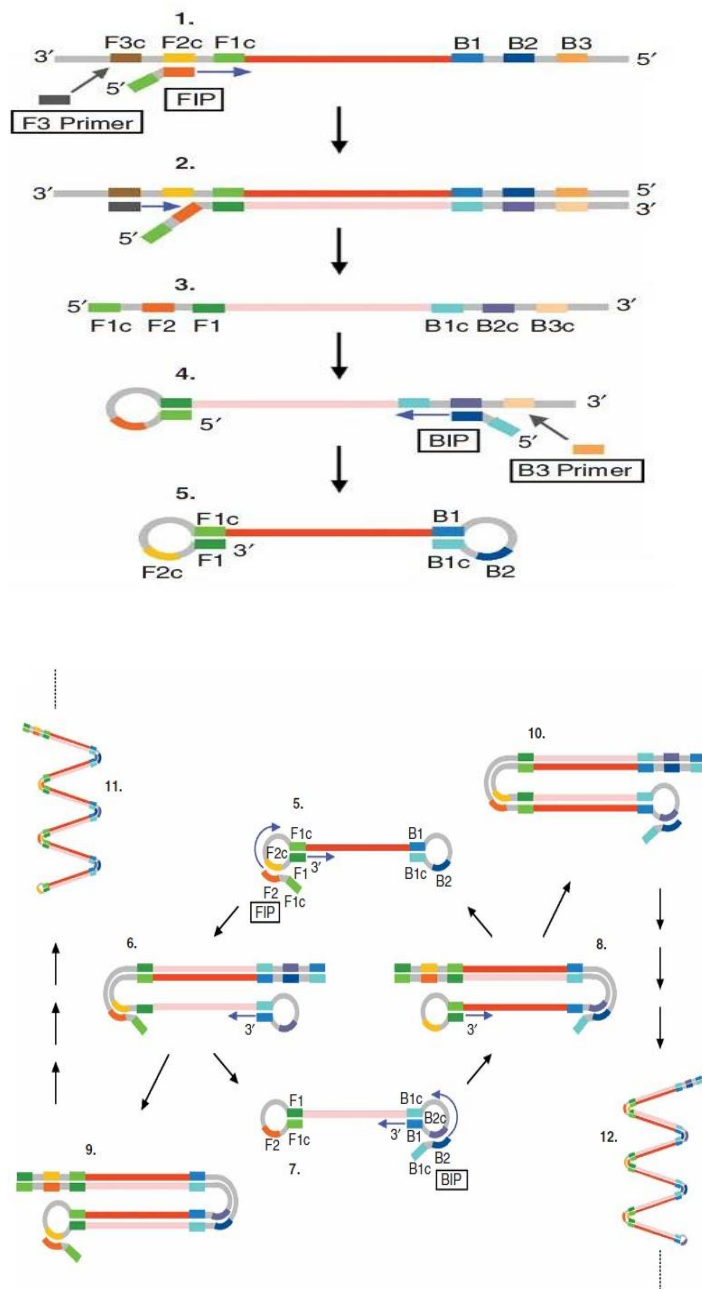


Figure 2.2: The LAMP principle. Starting material producing step (1-5), Cycling amplification step (5-8) and Elongation step (9-12) (Eiken Chemical Co. Ltd, 2005)

2.4 *Entamoeba histolytica* genome

Entamoeba histolytica draft genome was initially published by Loftus et al. (2005). They reported *E. histolytica* genome size as ~24 Mbps, split into 14 chromosomes, 9,938 predicted genes averaging 1.17 kilobases (kb) in size and comprising 49% of the genome.

After five years from the initial submission of the *E. histolytica* draft genome publication, Lorenzi et al., re-examined the original ~24 Mbps assembly and the annotation of the predicted genes. The evaluation of the genomic sequence led to the identification of more than one hundred artifactual tandem duplications that were eliminated by re-assembling the genome. The reannotation was done using a combination of manual and automated genome analysis. The new 20 Mbps assembly contains 1,496 scaffolds and 8,201 predicted genes, of which 60% are identical to the initial annotation and the remaining 40% underwent structural changes. Functional classification of 60% of the genes was modified based on recent sequence comparisons and new experimental data. Putative function was assigned to 3,788 proteins (46% of the predicted proteome) based on the annotation of predicted gene families, and 58 protein families of five or more members that share no homology with known proteins and thus could be *entamoeba* specific identified. Genome analysis also revealed new features such as the presence of segmental duplications of up to 16 kb flanked by inverted repeats, and the tight association of some gene families with transposable elements (Lorenzi et al., 2010). *E.histolytica* genome has GC content of 24.2% (Lorenzi et al., 2010).

2.5 Hemolysin (HLY6) gene

The hemolysin gene, HLY6, is encoded on the antisense strand of the large subunit ribosomal RNA (3,465 bps and 33.9% GC content) in the two inverted repeats of rDNA located on the 25 kbps palindromic circular episome of *E. histolytica* (Ramachandran *et al.*, 1993; Bhattacharya *et al.*, 1989; Huber *et al.*, 1989). The open reading frame of the HLY6 gene is conserved and multi-copy (400 copies/cell) with hemolytic activity (Rivera & Ong, 2013). The high copy number of HLY6 gene in *E. histolytica* genome makes it ideal for LAMP due to more regions of DNA to which the primers can hybridize (Liang *et al.*, 2009). In addition, primers specific for the HLY6 gene have already been used as tools for specific identification of *E. histolytica* in liver abscess and fecal samples (Zindrou *et al* 2001; Rivera & Ong 2013).

2.6 18S rRNA gene

The *E. histolytica* rRNA genes are present on an extrachromosomal, circular plasmid which is 25 kbps in size and present in about 200 copies per cell. 18S ribosomal DNA is conserved and has 1,946 bps (Ramachandran *et al.*, 1993).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design and location

This was a quantitative experimental laboratory study carried out at KEMRI, Center for Microbiology Research Parasitology laboratory. Briefly, one time sampling of stool specimens was done from patients aged 5 years and over and, presenting with diarrhea, abdominal pain and/or discomfort. Data such as sample ID, sample source, date of collection and microscopy status was captured in tables. A modified LAMP test for amoebiasis was developed and compared with published LAMP and PCR tests.

Samples for test evaluation were collected from three health centres in Mukuru informal settlements and one inpatient pediatric ward at Mbagathi District Hospital.

3.2 Sample size determination

The formula to work out the sample size was borrowed from the textbook *Sampling methods* by William G. Cochran (Cochran, 1977):

$$n_0 = t^2 pq / d^2$$

Where:

n_0 is the sample size

t is the value for the selected alpha level, 1.96

p is the estimated proportion of an attribute that is present in the population (prevalence)
= 32% (0.32) (Kagira *et al.*, 2011)

q is 1 - p = 0.68

(p)(q) are the estimate of variance

d is the acceptable margin of error, 0.05

$n_0 = 1.96^2 (0.32) (0.68) / 0.05^2 = 334$ samples

All positive samples as per microscopy analysis were used.

3.3 Clinical samples

Three hundred and thirty four samples were collected, and examined for the presence of *E. histolytica*, from children who presented to three participating outpatient clinics at Mukuru informal settlements i.e Medical Missionaries of Mary clinic, Reuben Centre clinic, City council clinic and those admitted to the paediatric ward of Mbagathi District hospital, Nairobi. The stool specimens were collected from patients who were 5 years of age and over and, presenting with diarrhea, abdominal pain and/or discomfort. Specimens were collected in sterile and clean capped containers and transported to the Centre for Microbiology Research, Parasitology laboratory. Stool samples were stored at -20°C and their aliquots at 4°C preserved in 2.5% potassium dichromate. In order to

improve sensitivity of microscopy in detection of *E. histolytica* cysts, the technique of formal-ether concentration was applied (Cheesbrough, 2005).

3.4 DNA Extraction

The DNA was prepared from 126 samples scored as positive for *Entamoeba* (*E. histolytica*, *E. dispar* and *E. moshkovskii* complex) using microscopy. Genomic DNA was extracted using QiAmp[®] DNA stool Mini kit (Qiagen, Crawley, West Sussex, United Kingdom) as per the manufacturer's instructions with slight modifications. Briefly, 200 µl of fecal suspension was washed five times with distilled water. To this suspension, 1.4 ml of ASL buffer was added and subjected to five times thawing (80°C) and freezing (-80°C) to rupture the rigid cysts. The genomic DNA was eluted in 50 µl nuclease-free water and stored at -20°C until use.

3.5 Cultivation of *E. histolytica*

Locke-egg slant media was used to cultivate *E. histolytica* (Clark & Diamond, 2002) with some modifications. The media was prepared by mixing Locke's solution and fresh hens' eggs. Locke's solution was prepared by dissolving in one litre of distilled water 8g sodium chloride, 0.2g calcium chloride, 0.2g potassium chloride, 0.01g magnesium chloride, 2g sodium phosphate, dibasic, 0.4g sodium bicarbonate and 0.3g potassium phosphate, monobasic (Rankem, India). The solution was autoclaved for 15 minutes at 121°C and 15 lbs and cooled to 25°C. The egg slants were prepared by emulsifying 45ml egg in 12.5 ml Locke's solution and pouring 5 ml of emulsified egg to 16 x 125 mm culture tubes; the tubes were slanted at an angle (~30°) to produced 12-15 mm butt, then

autoclaved at 100°C for 10 minutes and cooled to 25°C. The slants were then overlaid with 6 ml Locke's solution and autoclaved at 121°C, 15 lbs for 15 minutes, cooled to 25°C then refrigerated at 4°C until use.

To prepare for inoculation, 0.2 ml of 5mg/ml rice starch (Oxford Labchem, India) and 1,375 µl heat-inactivated adult bovine serum was added to each Locke-egg media tube. 0.25 ml emulsified *Entamoeba* positive stool sample was inoculated into the enriched media. Seven hundred-µl mineral oil was overlaid on culture medium surface in duplicate tubes to provide micro-aerophilic environment. The inoculated tubes were incubated at 35.5°C for 48 hours.

3.6 Polymerase Chain Reaction test

The polymerase chain reaction (PCR) test targeting the small-subunit rRNA gene was used (Hamzah *et al.*, 2006) with some modifications. Briefly, a 25µl test was done and consisted of 1× PCR buffer, 1.5 mM MgCl₂, 2 mM dNTPs, 0.5 U of *Taq* polymerase and 10 pmol of forward primer (EntaF) and reverse primer (EhR). The primer sequences are 5'-ATG CAC GAG AGC GAA AGC AT- 3' (EntaF) and 5'-GAT CTA GAA ACA ATG CTT CTC T- 3' (EhR) (Hamzah *et al.*, 2006). These primers generate a 166-bp PCR product and are specific for *E. histolytica*. The reference DNA template was 2µl of DNA and 3-4ul for clinical samples. The amplifications were done in a PCR system 9700 thermal cycler (Applied Biosystems, UK) under the following cycling conditions: An initial denaturation step at 94 °C for 3 minutes, followed by 35 cycles each consisting denaturation at 94 °C for 1 minute, annealing at 58 °C for 1 minute and

extension at 72 °C for 1 minute. The final extension was at 72 °C for 7 minutes. Reactions were done in duplicates and the resulting amplification products were separated by electrophoresis in 2.0% agarose gel in 1 × Tris-borate-EDTA at 100 V for 45 minutes and visualized under UV light after staining with ethidium bromide.

3.7 Design of LAMP primers

Entamoeba histolytica 18S small subunit ribosomal RNA (18S rRNA) (Genbank accession number X64142) and hemolysin (HLY6) gene (GenBank accession number Z29969.1) sequences were extracted from genbank (<https://www.ncbi.nlm.nih.gov/genbank/>). They were blasted using the basic local alignment search tool (<http://www.ncbi.nlm.nih.gov/BLAST>) to ensure specificity to the *E. histolytica* genome. Four sets of primers each recognizing ten distinct sections of *E. histolytica* 18S small subunit ribosomal RNA (18S rRNA) and hemolysin (HLY6) gene were designed using Primer Explorer version 3 software (<http://primerexplorer.jp/lamp3.0.0/index.html>). The targets were chosen due to the reported specificity and high number of copies (~200 copies) for rRNA (Huber *et al.*, 1989) and HLY6 (400 copies/cell) (Rivera & Ong, 2013). The software designed the following primers: forward and backward outer primers (F3 and B3) and forward and backward inner primers (FIP and BIP). The loop forward and backward primers (LF and LB) and stem forward and backwards primers (SF and SB) were manually designed following the respective published primer characteristics (Notomi *et al.*, 2000; Gandelman *et al.*, 2011). The following was considered during design of the manual primers: (i) 18-30 bases long, (ii) Contain no internal secondary structure, (iii) G/C

content between 40% and 60% (iii) balanced distribution of G/C and A/T rich domains (iv) not complementary to each other at their 3' ends and are not self complementary and (v) a melting temperature (T_m) that allows annealing to occur between 55° and 65°C.

The formulae $T_m=2(A+T) + 4(G+C)$ was used to calculate the primers' melting temperature. Manual manipulation of HLY6 primers to adhere to set melting temperature range was carried out. The primers were blasted for target specificity using the basic local alignment search tool (<http://www.ncbi.nlm.nih.gov/BLAST>). The designed tests consisted of F3/B3, FIP/BIP, LF/LB and SF/SB primer combination.

3.8 LAMP reactions

The 18S and HLY6 LAMP primers were first analyzed for detection of the reference *E. histolytica* HM-1: IMSS using standard LAMP conditions. The tests specificity was checked with closely associated pathogen DNA extracted from morphologically similar but non-pathogenic *Entamoeba dispar* and *Giardia lamblia*. The primer set(s) that passed this criteria were then analyzed using a 10-fold serial dilution of control DNA using the standard LAMP test conditions (Notomi *et al.*, 2000). The most sensitive primer set for each target was selected for further analysis. The new tests were labeled stem 18S and stem HLY6 LAMP tests respectively and the selected primer sets (Table 4.1) were used to optimize respective LAMP test using Taguchi method (Cobb & Clarkson, 1994). Briefly, four reaction components determined to have the greatest effect on LAMP reaction namely inner primers, loop primers, stem primers and dNTPs had their concentrations varied at three levels. The inner primer concentration was

varied from 30 to 60 pmol, loop primers from 10 to 30 pmol, stem primers from 10 to 40 pmol and dNTPs from 1 to 3 mM respectively. The concentrations of each reaction component were arranged in an orthogonal array and used to determine the amount of amplification product formed (Cobb & Clarkson, 1994). This was followed by regression analysis to determine the concentration optima for each selected reaction component (Cobb & Clarkson, 1994).

Other reaction components included 1× ThermoPol reaction buffer contained 20mM Tris-HCl (pH8.8), 10mM KCl, 10mM (NH₄)₂SO₄, 2mM MgSO₄ and 0.1% Triton X-100. The *Bst* 3.0 DNA polymerase (New England Biolabs, MA USA) was 0.5µl and SYTO-9 fluorescence dye at 2.0 µM (Molecular Probes, Oregon, USA). The template was 2µl of DNA. The LAMP reaction were performed for 60 minutes at 62°C using the real time PCR machine and data acquired on FAM channel followed by reaction inactivation at 80°C for 5 minutes. Once the optimized reaction conditions were determined the reactions were duplicated using a thermocycler and a water bath that maintained temperature at ~61-63°C. The template for clinical samples was varied from 2-4 µl. For comparative purposes, the published small subunit rRNA LAMP (Liang *et al.*, 2009) and HLY6 LAMP (Rivera & Ong, 2013) tests were included.

3.9 Analytical sensitivity LAMP test

The analytical sensitivity for the stem 18S LAMP test was carried out in duplicates using a 10-fold serial dilution of ~300 µg/ml of reference DNA from *E. histolytica* DNA. To cover different published LAMP tests combinations (Liang *et al.*, 2009;

Gandelman *et al.*, 2011; Rivera & Ong, 2013) the 18S LAMP test was analyzed in the following combinations: [i] Stem LAMP test with outer primers, [ii] Stem LAMP test without outer primers, [iii] Standard LAMP with loop primers and [iv] Standard LAMP test without loop primers (Table 4.2). These formats were compared using 10-fold serial dilution with published LAMP test (Liang *et al.*, 2009) and PCR targeting 18S rRNA (Hamzah *et al.*, 2006).

3.10 Detection and confirmation of LAMP product

The LAMP product was detected through fluorescence of SYTO-9 dye in real time PCR machine, through electrophoresis in 2% agarose gel stained with ethidium bromide and after addition of 1µl of 1/10 dilution of 10,000× stock SYBR® Green I (Sigma-Aldrich, St. Louis, MO, USA). To confirm that *E. histolytica* LAMP test amplified the predicted product, melt peaks were acquired using 1°C steps, with a hold of 30 s, from 62°C to 96°C (Monis *et al.*, 2005) post amplification and through digestion of the resulting LAMP product using restriction enzyme *DdeI* (New England BioLabs, MA, USA) and following manufacturers recommendations.

3.11 Data management and analysis

The collected samples were recorded in tables showing sample ID, sample source, date of collection and microscopy status. The tests data was entered in Excel sheet version 2007. Microsoft Office Excel 2007 software was used to capture the data. Variables such as sample source, sample ID, date of collection, microscopy status, PCR status and LAMP tests status were captured in the database. Images of gel electrophoresis were

documented using Canon digital camera G1X mounted on Benchtop 2UV Transilluminator (UVP, LLC, USA). The bioinformatics tool, BLAST, which uses the chosen BLAST algorithm was used for similarity searches. The Expect value (E value) and percentage identity were used to determine the best alignments.

The items analyzed included the test analytical sensitivity and specificity for known controls. The sensitivity analysis indicated the lowest levels of detection as compared to the published LAMP test and PCR. Tests were done in triplicates to check for reproducibility.

Kappa coefficient as described by Viera and Garrett (2005) was used to determine agreement among the molecular tests and between new stem LAMP and microscopy. Kappa coefficient was calculated using the online statistical program <http://graphpad.com/quickcalcs/kappa2/>

3.12 Reference DNA

To standardize the test, reference DNA was required. The reference DNA sample of *Entamoeba histolytica* HM-1: IMSS was kindly provided by Dr. Graham Clark, Department of Pathogen Molecular Biology, London School of Hygiene and Tropical Medicine, UK. The DNA to check the test specificity was prepared from *Entamoeba dispar* and *Giardia lamblia* using commercial DNA extraction kit (Qiagen, Essex, UK).

3.13 Ethical clearance

Approval for the study was sort from, and given by the KEMRI Scientific and Ethics Review Unit (SSC No. 2834) (Appendix 1). All parents and/or guardians of participating children were informed of the study objectives and voluntary written consent was sought and obtained before inclusion. A copy of the signed consent was filed and stored in password-protected cabinets at KEMRI. The samples were analysed anonymously.

CHAPTER FOUR

RESULTS

4.1 LAMP primers

Four sets of primers for each target gene (18S rRNA and HLY6 genes) were designed (Table 4.1). All primers blasted for target specificity gave 100% identity to *E. histolytica* genome sequence.

Table 4.1: Nucleotide sequences for *E. histolytica* primers for stem 18S rRNA and HLY6 LAMP tests.

Target	Primer name	Sequence (5'-3')	Bases	Final Amplicon size
18S rRNA	F3	AAATACAAGGATAGCTTTGTG	21	207
	B3	AAGCTCCCTCTCCGATGTC	19	
	FIP	CTCAATTCATTGAATGAATTGGCATGATAAAGATAATACTTGAGAC	46	
	BIP	CAATGAGAATTTCTGATCTATCCGTTATCCGTTATAATCTTGG	43	
	LF	TTTGTACTAATACAAACTGGATC	23	
	LB	CAGTTGGTAGTATCGAGGAC	20	
	SF	CGACAATTGTAGAACACACAG	21	
	SB	ATCCTAACTCACTTAGAATGTC	22	
HLY6	F3	ATACTTGAACGGATTG AAGCC	21	190
	B3	GTTTATTCATATGTTTGACAAGA	22	
	FIP	CGCCCTATACTCAAATATGACACTTTGGTGGAAGATTCACG	41	
	BIP	AGGAAGGTCAAAGTATTAATAGTGTGAGTGAATATACTCACC	43	
	LF	GTAATTTGCACGTTAACACTG	21	
	LB	TGGTAAAGATAATGATTAGGTG	22	
	SF	CTGGTTCCACCTGAATATTC	20	
	SB	TACTAGATAGTTCGATGAGTC	21	

Table 4.1: Nucleotide sequences for *E. histolytica* primers for stem 18S rRNA and HLY6 LAMP tests.

Target	Primer name	Sequence (5'-3')	Bases	Final Amplicon size
18S rRNA	F3	AAATACAAGGATAGCTTTGTG	21	207
	B3	AAGCTCCCTCTCCGATGTC	19	
	FIP	CTCAATTCATTGAATGAATTGGCATGATAAAGATAATACTTGAGAC	46	
	BIP	CAATGAGAATTTCTGATCTATCCGTTATCCGTTATAATCTTGG	43	
	LF	TTGTACTAATACAAACTGGATC	23	
	LB	CAGTTGGTAGTATCGAGGAC	20	
	SF	CGACAATTGTAGAACACACAG	21	
	SB	ATCCTAACTCACTTAGAATGTC	22	
HLY6	F3	ATACTTGAACGGATTG AAGCC	21	190
	B3	GTTTATTCATATGTTTGACAAGA	22	
	FIP	CGCCCTATACTCAAATATGACACTTTGGTGGAAGATTCACG	41	
	BIP	AGGAAGGTCAAAGTATTAATAGTGTTGAGTGAATATACTCACC	43	
	LF	GTAATTTGCACGTTAACTG	21	
	LB	TGGTAAAGATAATGATTAGGTG	22	
	SF	CTGGTTCCACCTGAATATTC	20	
	SB	TACTAGATAGTTCGATGAGTC	21	

4.2 *Entamoeba histolytica* LAMP optimum reaction conditions

The Taguchi method determined the optimal concentrations for the four reaction components in stem 18S LAMP test as 35pmol for FIP/BIP, 18pmol for loop primers, 23pmol for stem primers and 2mM dNTPs. The stem HLY6 LAMP test showed the most efficient reaction at 40pmol for FIP/BIP, loop primers at 20pmol, stem primers at 15pmol and 1.5mM dNTPs. Concentrations for other reagents were as reported previously (Notomi *et al.*, 2000). The optimum temperature for stem LAMP test was determined at 62⁰C and 50 minutes being the reaction cut-off point (Figure 4.1). Stem18S LAMP test indicated higher (~30pg/ml) sensitivity to stem HLY6 LAMP test (~3µg/ml) hence the latter was not progressed in the analysis of clinical samples.

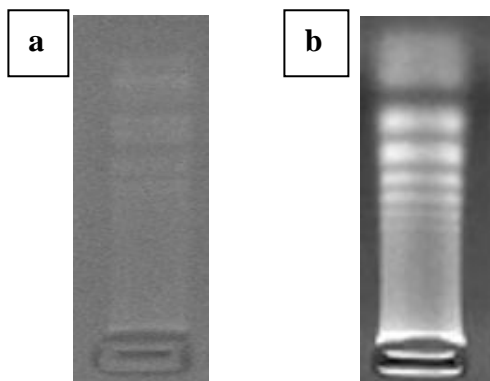


Figure 4.1: Optimization of stem 18S *E. histolytica* LAMP test reactions. (a) Gel image of LAMP reaction at 40 minutes. The faint band is due to inadequate product formation. (b) LAMP reaction at 50 minutes.

4.3 Analytical sensitivity of LAMP and PCR tests

The stem 18S LAMP tests (with and without outer primers) indicated identical detection limit of 10^{-7} (30 pg/ml) (Figure. 4.2a; Table 4.2) while the standard LAMP test (with loop primers) and published LAMP test (without loop primers) indicated detection levels ranging from 30 to 300 pg/ml (Table 4.2). The standard LAMP test (without loop primers) showed low sensitivity and was not included for further analysis (Table 4.2). The stem 18S LAMP test sensitivity was not altered when the stem primers were used either in their forward or reverse orientation and/or when the template concentration was increased. The PCR test based on the same target showed detection limit of 10^{-5} (3 ng/ml) (Figure. 4.2b; figure 4.3). The stem 18S LAMP test sensitivity was reproducible using thermocycler and water bath and no cross reactivity was recorded with non-target DNA (Figure 4.4). The optimized *E. histolytica* stem 18S LAMP test with and without outer primers F3 and B3 showed reduction in reaction time (cycle threshold = *CT*) value of ~11 cycles (Table 4.2) compared to the standard LAMP test targeting the same gene.

Table 4.2: The analytical sensitivity of 18S rRNA LAMP test combinations and PCR test using a 10-fold serial dilution of *E. histolytica* DNA

Test	Combination	10-fold serial dilution						<i>C_T</i> value [†]	Ref
		Neat	10 ^{-1 to 4}	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸		
Stem LAMP ^a	F3/B3, FIP/BIP, LF/LB, SF/SB	+	+	+	+	+	-	28	
Stem LAMP ^b	FIP/BIP, LF/LB, SF/SB	+	+	+	+	+	-	28	
Standard LAMP ^c	F3/B3, FIP/BIP, LF/LB	+	+	+	+	-	-	33	
Standard LAMP ^d	F3/B3, FIP/BIP	+	+	±	-	-	-	-	
Published LAMP ^e	F3/B3, FIP/BIP	+	+	+	-	-	-	39	Liang <i>et al.</i> , 2009
PCR test	EntaF and EhR	+	+	+	-	-	-	nd	Hamzah <i>et al.</i> , 2006

^a LAMP test with outer F3/B3 primers

^b LAMP without outer F3/B3 primers (amplicons were less bright)

^c Standard LAMP test (the most common LAMP format)

^d Standard LAMP test format without loop primers (initial format, not commonly used)

^e Published LAMP test without Loop primers

[†] The cycle threshold (*C_T*) in minutes for 10-fold dilution of 10⁻⁵

The lower the value the greater the amount of target DNA formed (in italics)

± Half of the replicates were positive (2 out of 4)

nd = not done

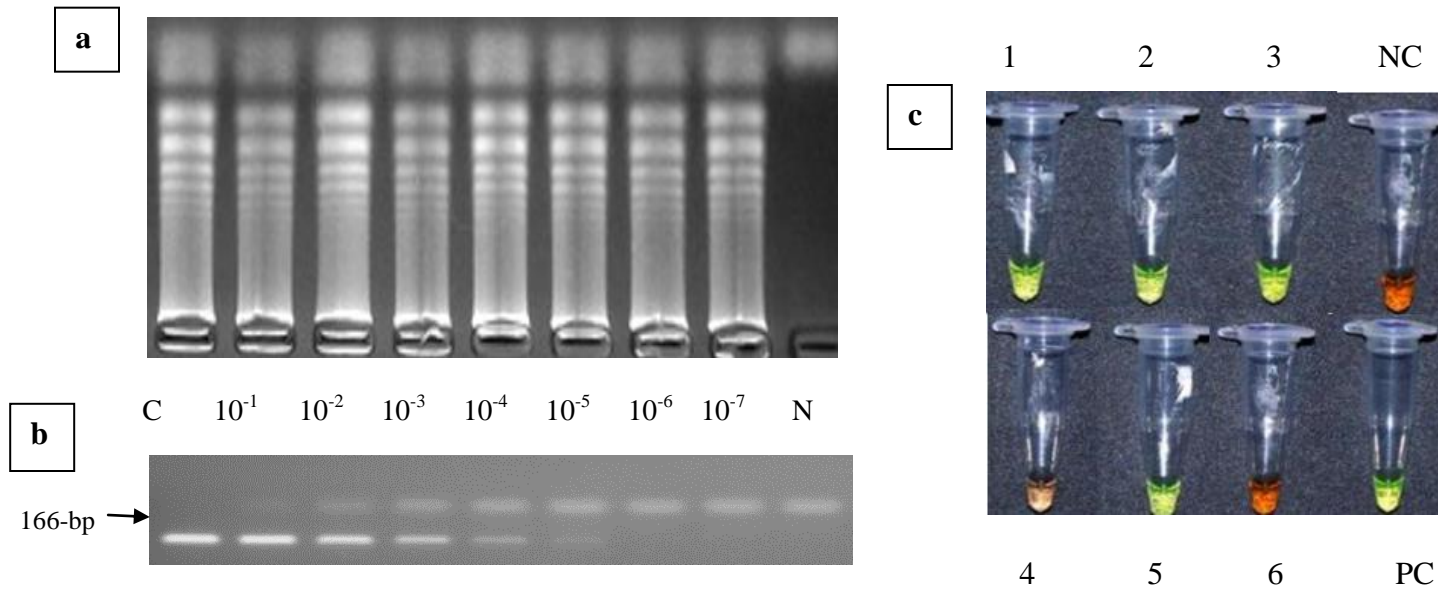


Figure 4.2: Sensitivity and specificity analysis. (a) The sensitivity of the stem18S LAMP test with outer primers using a 10-fold serial dilution of *E. histolytica* DNA. (b). The PCR sensitivity levels and showing the 166 bp amplicon. (c) The visual appearance of stem LAMP test amplification product after addition of 1/10 dilution of SYBR® Green I dye. The dye fluoresces strongly when bound to the double stranded DNA and the resulting DNA-dye-complex gives a green colour while fluorescence is minimal when the dye is free in the solution and gives orange/brown colour. Samples 1, 2, 3 and 5 are *E. histolytica* positive and samples 4 and 6 are negative samples. PC = positive control and NC = negative control

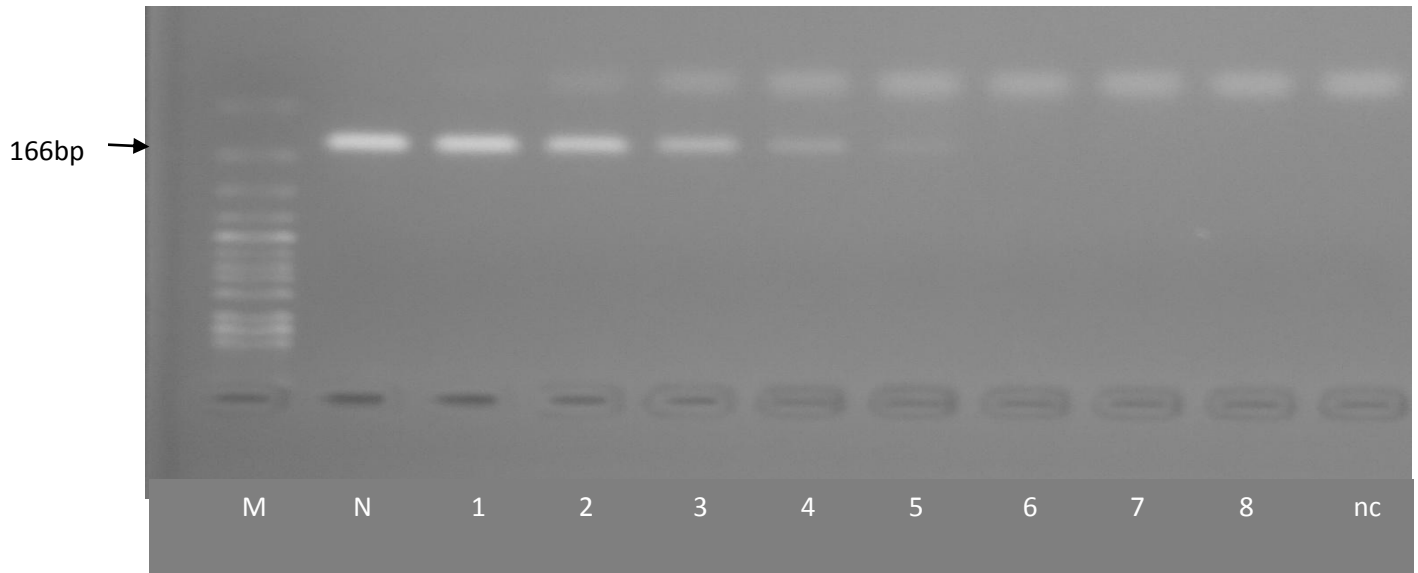


Figure 4.3: Sensitivity of PCR test. A 10-fold serial dilution of *E. histolytica* DNA analyzed using published primers (Hamzah *et al.*, 2006). Target gene: small-subunit rRNA. Expected product size is 166bp. M: Molecular weight marker (100bp ladder), N: Neat DNA, 1: 10^{-1} , 2: 10^{-2} , 3: 10^{-3} , 4: 10^{-4} , 5: 10^{-5} , 6: 10^{-6} , 7: 10^{-7} , 10^{-8} , nc- Negative control.

E. h E. d G. l nc

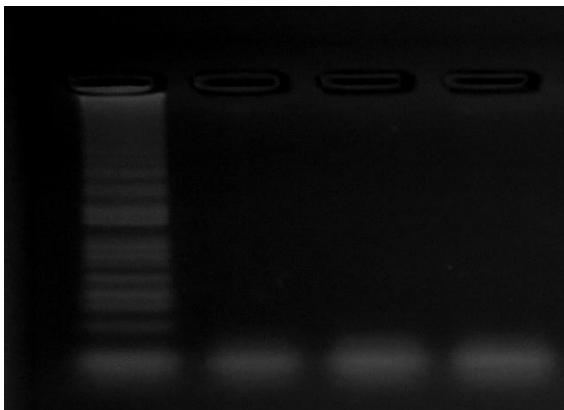


Figure 4.4: Specificity of the stem 18S LAMP assay. No amplification product was detected when DNA from *E. dispar* and *G. lamblia* was used. E.h - *E. histolytica*, E.d- *E. dispar*, G.l- *G. lamblia*, nc- negative control.

4.4 *Entamoeba histolytica* LAMP product

The optimized *E. histolytica* stem 18S LAMP tests with and without outer primers indicated similar exponential real time amplification curves (Figure 4.5a) with post amplification melting temperature (T_m) of $\sim 86^\circ\text{C}$ (Figure 4.5b). The LAMP products showed the predicted ladder like pattern on the agarose gel indicating the formation of stem-loop with inverted repeats (Figure 4.2a). On addition of SYBR® Green I, the positive product turned green and the negative ones remained orange (Figure 4.2c). The *DdeI* restriction enzyme digestion of stem 18S LAMP test product, which cuts between primers F2 and B2c, indicated the predicted amplicons of 143 bp and 103 bp (Figure 4.6).

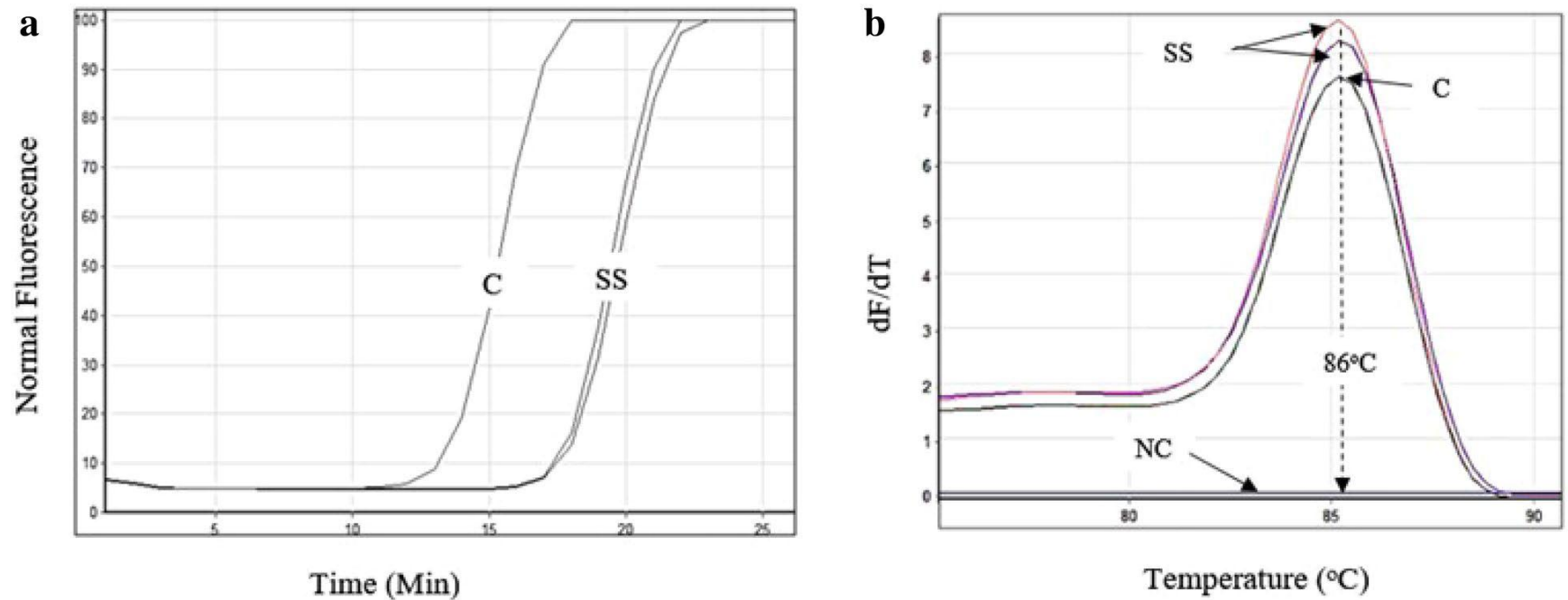


Figure 4.5: (a) The real time curves acquired using *E. histolytica* stem 18S-LAMP test as monitored using the real time PCR machine. The stem-LAMP tests with and without outer primers F3 and B3 showed similar amplification curves. (b) The *E. histolytica* melt peaks acquired post amplification on the FAM channel. The positive clinical samples showed identical T_m of $\sim 86^\circ\text{C}$ with the reference DNA indicating identical amplicons. C = positive control DNA, SS = two samples using PCR and NC = negative control. dF/dT = fluorescence.

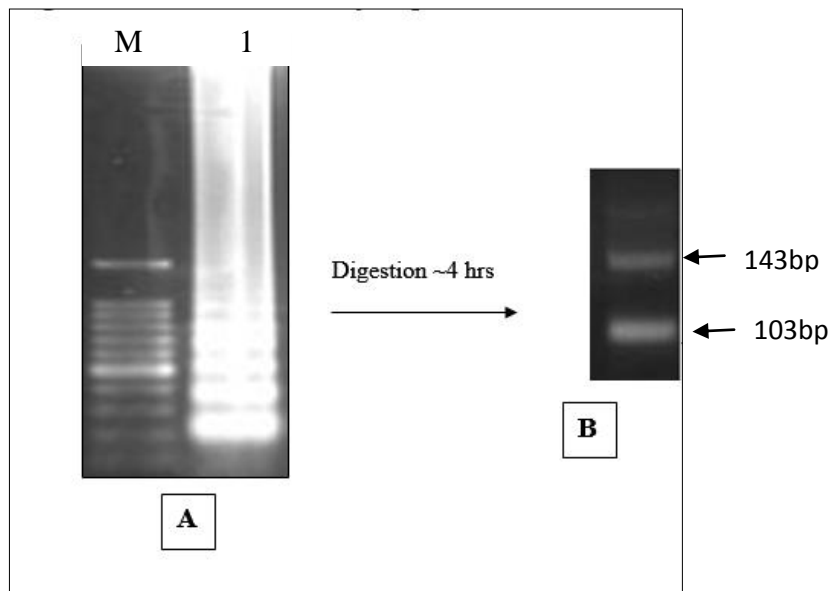


Figure 4.6: DdeI restriction enzyme digestion of stem 18S LAMP test product. (A) LAMP product. M = 100 bp DNA marker, 1 = LAMP product. **(B)** *DdeI* restriction enzyme product at 143 bp and 103 bp.

4.5 Results for clinical samples

One hundred and twenty six samples were scored positive for *entamoeba* using microscopic examination. The stem 18S LAMP tests with and without outer primers detected 36 (28.6%) while the standard LAMP tests with and without loop primers detected 26 (20.6%) and 20 (15.9%) of *E. histolytica* DNA respectively (Table 4.3). We recorded intermittent non-specific products with some replicates for stem LAMP test with outer primers. The conventional PCR identified 18 (14.3%) as *E. histolytica*. Other LAMP tests formats were not used in sample analysis since they indicated lower (3µg/ml) analytical sensitivity. Statistical analysis using Kappa statistics of the Stem-18S LAMP, Standard LAMP and PCR and microscopy tests indicated fair to good agreement (Table 4.4).

Table 4.3: Comparative evaluation of Stem-18S LAMP, Standard LAMP and PCR tests in detection of *E. histolytica* DNA in clinical samples (n = 126).

Type of Test	Accelerating primers	No. Positive	Reference
Stem 18S LAMP	Loop and Stem	36(28.6%)	This study
Standard LAMP	Loop	26(20.6%)	This study
Published LAMP	-	20(15.9%)	Liang <i>et al.</i> (2009)
PCR [†]	n/a	18(14.3%)	Hamzah <i>et al.</i> (2006)

[†] PCR positive samples were positive using all LAMP formats.

Table 4.4: Comparative analysis of the molecular-based methods in the detection of *E. histolytica* DNA in clinical samples using Kappa Coefficient.

Type of Tests	Kappa coefficient	Standard Error of Kappa	95% confidence interval	Strength of agreement
Stem LAMP Vs Published LAMP	0.641	0.078	0.488 to 0.794	good
Stem LAMP Vs Published PCR	0.588	0.082	0.428 to 0.749	moderate
Published LAMP Vs Published PCR	0.938	0.043	0.853 to 1.000	Very good
Microscopy (gold standard) Vs Stem LAMP	0.222	0.045	0.245 to 0.420	fair

Kappa	Agreement
< 0	Less than chance agreement
0.01–0.20	Slight agreement
0.21– 0.40	Fair agreement
0.41–0.60	Moderate agreement
0.61–0.80	Substantial agreement
0.81–0.99	Almost perfect agreement

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

In the present study, a rapid and visual LAMP assay for detection of *Entamoeba histolytica* was developed. The stem 18S LAMP test is a modification of the standard LAMP test through inclusion of stem primers and indicate higher (~30pg/ml) analytical sensitivity and shorter (50 minutes) reaction time to results which translate to a higher detection of pathogen DNA in clinical samples compared to the standard LAMP format. The recorded superior sensitivity can be attributed to the multiplexing of reaction accelerating primers (loop and stem primers) in a single reaction compared to the standard LAMP formats which have no reaction accelerating primers or relies on loop primers only. The loop primers are known to accelerate the reaction by priming the sequence loops between FIP/BIP primers (Nagamine *et al.*, 2002) while the stem primers accelerate reaction by targeting the stem section of the sequence (Gandelman *et al.*, 2011). It is therefore the use of two accelerating primer sets that exponentially increase the amount of LAMP product hence reduction in reaction time. Standard LAMP and PCR take 60 min and ~100 min to results respectively (Notomi *et al.*, 2000; Hamzah *et al.*, 2006).

Surprisingly the omission of outer primers did not affect the stem 18S LAMP test sensitivity, although the ladder like bands on agarose gel were less bright compared with the format with the outer primers. This may indicate formation of less product in the latter format but did not translate to less sensitivity in terms of pathogen DNA detection.

Indeed, the products of the two LAMP formats were confirmed to be identical through acquisition of post amplification melt curves (Figure. 4.5) and through digestion of the product with restriction enzyme (Figure 4.6).

The primary role of the outer primers is to displace the newly synthesized strands into a single strand making it available for extension by either inner primer (Notomi *et al.*, 2000) and therefore outer primers do not form part of the final LAMP product. It appears that the inner, loop and stem primers may have some strand displacement activity, although not as efficient as the outer primers. The possibility of omitting the outer primers gives more flexibility for additional of more reaction accelerating primers.

It is not clear as to why the LAMP test based on the HLY6 gene showed low sensitivity (10^{-2} , 3 μ g/ml) and low detection of PCR positive samples despite the reported higher number of copies (~400 copies) (Rivera & Ong, 2013). The lower sensitivity of the standard LAMP test i.e. the published format (Liang *et al.*, 2009) compared to stem LAMP format is attributable to absence of loop primers in the standard format. Indeed this study's identical LAMP format based on the same gene showed similar lower detection levels (Table 4.2). On addition of loop primers, the analytical sensitivity improves by 10-fold and translate to detection of more positive clinical samples (Table 4.3). The use of loop primers to accelerate LAMP tests is recommended (Nagamine *et al.*, 2002) and has been demonstrated to significantly improve LAMP tests sensitivity and detection of pathogen DNA in clinical samples (Njiru *et al.*, 2008; Njiru *et al.*, 2012). The sensitivity of *E. histolytica* LAMP test is further improved in this study through multiplexing loop primers with stem primers. This sequential addition of

primers resulting in improvement of LAMP test sensitivity is unequivocal demonstration that the reaction accelerating primers are critical to any successful LAMP test. The resulting product was easily detected using SYBR Green I allowing visual inspection of results. The SYBR Green I is cheap but the need to open the tube to add the dye risk contamination with amplicon. Further the dye is non-specific and binds to any double stranded DNA including primer-dimers. To increase the confidence of using non-specific dyes, rigorous test optimization is necessary to reduce formation of spurious products. In addition, the use of more negative controls is recommended to increase the confidence limit.

The stem LAMP test classified 36 (28.6%) of 126 DNA samples as *E. histolytica*. All the PCR positive samples were also positive with the new LAMP test. In this study, the detection of *E. histolytica* was higher than prevalence of 13.3% (Hamzah *et al.*, 2006) and 3.3% (Zeyrek *et al.*, 2013) recorded earlier. This may be attributed to the improvement of the LAMP tests sensitivity reported here. All LAMP formats showed detection range of 15.9–28.6% which indicates LAMP method is superior to classical PCR and is a good improvement towards diagnosis of amoebiasis. Similar superior sensitivity of stem LAMP format to PCR has been recorded in diagnosis of sleeping sickness (Njiru *et al.*, 2017).

The cost of LAMP test is expected to be lower than that of PCR test as the former doesn't require expensive equipment such as a thermocycler. Further, the cost will continue to be lowered as ways of reducing the need for cold chain are researched on. For instance lyophilisation of LAMP recipe has been achieved for sleeping sickness

(Eiken, 2011). Previous studies indicated that LAMP reagents were stable at temperatures of up to 37°C (Thekiso *et al.*, 2009). Moreover, the kit should be affordable and stable under hot and humid tropical conditions (Njiru, 2012). More research is needed to generate LAMP reagents that are stable in extreme conditions.

This is the first study in Kenya to report the detection of *E. histolytica* using LAMP method. It is possible that the prevalence of *E. histolytica* is even higher since a large portion of samples remained un-identified or the microscopically observed cyst belong to the morphologically similar but non-pathogenic *Entamoeba dispar* and *moshkovskii*. No tests were done to check the presence of *E. dispar* and *E. moshkovskii*. The world prevalence of *E. dispar* is reported to be nine times that of *E. histolytica* (Markell *et al.*, 1999). If that phenomenon holds in the prevalence of this species in Kenya, then a large portion of the remaining 90 (71.4%) DNA could be *E. dispar*. Having methods that can accurately differentiate *Entamoeba spp.* is important to understand the prevalence of species in Kenya and avoid unnecessary chemotherapy in patients with non-pathogenic species.

Cultivation of *E. histolytica* was not successful. This was attributed to overgrowth of *Blastocystis hominis*, which consumed nutrients necessary for growth of *E. histolytica*. Due to time and cost constraints this option that was meant to provide enough *E. histolytica* parasites and consequently DNA to optimize various reactions in the study was not pursued. This did not affect results of the study.

It should be noted that in amoebiasis, the reason to treat is based on demonstration of trophozoites and/or cysts in the stool, as such LAMP test may not be relied upon to make a treatment decision. Since LAMP test is faster (50 minutes) to perform, the technique could form part of diagnostic algorithms for amoebiasis where LAMP test is used to select cases for further confirmation with PCR.

5.2 Conclusion

In this study:

- (i) A new stem 18S LAMP test, which is a modification of the standard LAMP test through inclusion of stem primers, was developed.
- (ii) The stem 18S LAMP test recorded higher (~30pg/ml) sensitivity and shorter (50 minutes) reaction time to results in comparison to the published PCR and LAMP tests. The detection of *E. histolytica* using the new test was higher (28.6%) than recorded earlier using PCR.

5.3 Recommendation

It is recommended that this new stem 18S LAMP test be further evaluated using a larger sample size and be part of diagnostic algorithms for amoebiasis.

REFERENCES

- Abd-Alla, M. D., Wahib, A. A., & Ravdin, J. I. (2000). Comparison of antigen-capture ELISA to stool-culture methods for the detection of asymptomatic *Entamoeba* species infection in Kafer Daoud, Egypt. *American Journal of Tropical Medicine and Hygiene*, *62*, 579–582.
- Acuna-Soto, R., Samuelson, J., Girolami, P., Zarate, L., Millan- Velasco, F., Schoolnick, G., & Wirth, D. (1993). Application of the polymerase chain reaction to the epidemiology of pathogenic and nonpathogenic *Entamoeba histolytica*. *American Journal of Tropical Medicine and Hygiene*, *48*, 58–70.
- Andrade, J. E., Mederos, R., Rivero, H., Sendzischew, M. A., Soaita, M., & Robinson, M. J. (2007). Amebiasis presenting as acute appendicitis. *South Medical Journal*, *100* (11), 1140-1142.
- Aonuma, H., Yoshimura, A., Perera, N., Shinzawa, N., Bando, H., & Oshiro, S., (2009). Loop-mediated isothermal amplification applied to filarial parasites detection in the mosquito vectors: *Dirofilaria immitis* as a study model. *Biomedical Central Parasites and Vectors*, *2*, 15.
- Blessmann, J., Van Linh, P., Nu, P. A., Thi, H. D., Muller-Myhsok, B., Buss, H., & Tannich, E. (2002). Epidemiology of amebiasis in a region of high incidence of amebic liver abscess in central Vietnam. *American Journal of Tropical Medicine and Hygiene*, *66*, 578–583.

- Centers for Disease Control and Prevention. (2009). Diagnostic and infective stages of *Entamoeba histolytica*. Retrieved from www.cdc.gov/dpdx/amebiasis/index.html
- Chahar, M., Mishra, N., Anvikar, A., Dixit, R., & Valecha, N. (2017). Establishment and application of a novel isothermal amplification assay for rapid detection of chloroquine resistance (K76T) in *Plasmodium falciparum*. *Science Representation*, 7, 41119.
- Cheesbrough, M. (2005). *District Laboratory Practice in Tropical Countries Part 1*. Cambridge: Press Syndicate.
- Clark, C. G., & Diamond, S. L. (2002). Methods for Cultivation of Luminal Parasitic Protists of Clinical Importance. *Clinical microbiology review*, 329–341.
- Cobb, H. & Clarkson, K. (1994). A simple procedure for optimising the polymerase chain reaction (PCR) using modified Taguchi methods. *Nucleic Acids Research*, 22, 3801-3805.
- Cochran, W. G. (1977). *Sampling techniques*, (3rd ed.). New York: John Wiley & Sons.
- Diamond, L. S., Clark, C. G., & Cunnick, C. C. (1995). YI-S, a casein-free medium for axenic cultivation of *Entamoeba histolytica*, related *Entamoeba*, *Giardia intestinalis* and *Trichomonas vaginalis*. *Journal of Eukaryotes Microbiology*, 42, 277–278.

- Diamond, L. S., Harlow, D. R., & Cunnick, C. C. (1978). A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 72, 431–432.
- Easton, A. V., Oliveira, R. G., O’Connell, E. M., Kepha, S., Mwandawiro, C. S., & Njenga, S. M. (2016). Multi-parallel qPCR provides increased sensitivity and diagnostic breadth for gastrointestinal parasites of humans: field-based inferences on the impact of mass deworming. *Parasites and Vectors*, 9, 38.
- Eiken Chemical Company Limited. (2005). The principle of LAMP method. Retrieved from loopamp.eiken.co.jp/e/lamp/index.html
- Eiken chemical Company Limited. (2011). Lyophilisation of LAMP recipe for sleeping sickness. Retrieved from <http://www.eiken.co.jp/en/news/pdf/20110916-1.pdf>
- Evangelopoulos, A., Legakis, N., & Vakalis, N. (2001). Microscopy, PCR and ELISA applied to the epidemiology of amoebiasis in Greece. *Parasitology International*, 50, 185–189.
- Evangelopoulos, A., Spanakos, G., Patsoula, E., Vakalis, N., & Legakis, N. (2000). A nested, multiplex, PCR assay for the simultaneous detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in faeces. *Annals of Tropical Medicine and Parasitology*, 94, 233–240.

- Fotedar, R., Stark, D., Beebe, N., Marriott, D., & EllisHarkness, J. (2007a). Laboratory Diagnostic Techniques for *Entamoeba* Species. *Clinical microbiology review*, 20(3), 511–532.
- Freitas, M. A., Vianna, E. N., Martins, A. S., Silva, E. F., Pesquero, J. L., & Gomes, M. A. (2004). A single step duplex PCR to distinguish *Entamoeba histolytica* from *Entamoeba dispar*. *Parasitology*, 128, 625–628.
- Furrows, S. J., Moody, A. H., & Chiodini, P. L. (2004). Comparison of PCR and antigen detection methods for diagnosis of *Entamoeba histolytica* infection. *Journal of Clinical Pathology*, 57, 264-266.
- Gachuhi, S., Obonyo, M., Odhiambo, R., Swierczewski, B., & Mwakubambanya, R. (2014). Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* Complex by Multiplex Polymerase Chain Reaction. *Scholars Academic Journal of Biosciences*, 2(11), 762-767.
- Gandelman, O., Jackson, R., Kiddle, G., & Tisi, L. (2011). Loop-mediated amplification accelerated by stem primers. *International Journal of Molecular Sciences*, 12, 9108-24.
- Gatei, W., Wamae, C. N., Mbae, C., Waruru, A., Mulinge, E., & Waithera, T. (2006). Cryptosporidiosis: Prevalence, genotype analysis, and symptoms associated with infections in children in Kenya. *American Journal of Tropical Medicine and Hygiene*, 75, 78-82.

- Gelderman, A. H., Bartgis, I. L., Keister, D. B. & Diamond, L. S. (1971). A comparison of genome sizes and therma-denaturation-derived base compositions of DNAs from several members of *Entamoeba* (histolytica group). *Journal of Parasitology*, 57, 912-916.
- Gonin, P., & Trudel, L. (2003). Detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* isolates in clinical samples by PCR and enzyme-linked immunosorbent assay. *Journal of Clinical Microbiology*, 41, 237–241.
- Gonzalez-Ruiz, A., Haque, R., Aguire, A., Castanon, G., Hall, A., Guhl, F., ... & Warhurst, D. C. (1994). Value of microscopy in the diagnosis of dysentery associated with invasive *Entamoeba histolytica*. *Journal of Clinical Pathology*, 47, 236–239.
- Hal, J. S., Stark, D. J., Fotedar, R., Marriott, D., Ellis, T. J., & Harkness, J. L. (2007). CLINICAL UPDATE: Amoebiasis: current status in Australia. *The Medical Journal of Australia*, 186, 8.
- Hamzah, Z., Petmitr, S., Mungthin, M., Leelayoova, S., & Chavalitshewinkoon-Petmitr, P. (2006). Differential detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* by a single-round PCR assay. *Journal of Clinical Microbiology*, 44(9), 3196-200.

- Haque, R., Ali, I. K., Akther, S., & Petri, W. A. Jr. (1998). Comparison of PCR, isoenzyme analysis, and antigen detection for diagnosis of *Entamoeba histolytica* infection. *Journal of Clinical Microbiology*, *36*, 449–452.
- Haque, R., Faruque, A. S. G., Hahn, P., Lyerly, D., & Petri, W.A. Jr. (1997). *Entamoeba histolytica* and *Entamoeba dispar* infection in children in Bangladesh. *Journal of Infectious Diseases*, *175*, 734–736.
- Haque, R., Mondal, D., Duggal, P., Kabir, M., Roy, S., & Farr, B. M. (2006). *Entamoeba histolytica* infection in children and protection from subsequent amebiasis. *Infection and Immunology*, *74*(2), 904-9.
- Haque, R., Neville, L. M., Hahn, P., & Petri, W. A. Jr. (1995). Rapid diagnosis of *Entamoeba* infection by using *Entamoeba* and *Entamoeba histolytica* stool antigen detection kits. *Journal of Clinical Microbiology*, *33*, 2558–2561.
- Herbinger, K. H., Fleischmann, E., Weber, C., Perona, P., Loscher, T., & Bretzel, G. (2011). Epidemiological, clinical, and diagnostic data on intestinal infections with *Entamoeba histolytica* and *Entamoeba dispar* among returning travelers. *Infection*, *39*, 527–535.
- Huber, M., Koller, B., Gitler, C., Mirelman, D., Revel, M., & Rozenblatt, S. (1989). *E. histolytica* ribosomal RNA genes are carried on palindromic circular DNA Molecules. *Molecular and Biochemical Parasitology*, *32*, 285-296.

- Huston, C. D., Haque, R., & Petri, W. A. Jr. (1999). Molecular-based diagnosis of *Entamoeba histolytica* infection. *Expert Reviews in Molecular Medicine*, 22, 1–11.
- Kagira, J. M., Maina, N., Njenga, J., Karanja, S. M., Karori, S. M., & Ngotho, J. M. (2011). Prevalence and Types of Coinfections in Sleeping Sickness Patients in Kenya (2000/2009). *Journal of Tropical Medicine*, 2011, 248914.
- Khairnar, K., & Parija, S. C. (2007). A novel nested multiplex polymerase chain reaction (PCR) assay for differential detection of *Entamoeba histolytica*, *E. moshkovskii* and *E. dispar* DNA in stool samples. *BioMedical Central Microbiology*, 7, 47.
- Klein, D. (2002). Quantification using real-time PCR technology: applications and limitations. *Trends in Molecular Medicine*, 8, 257–260.
- Lee, D., Kim, E. J., Kilgore, P. E., Takahashi, H., Ohnishi, M., Tomono, J., ... & Seki, M. (2016). A novel loop-mediated isothermal amplification assay for serogroup identification of *Neisseria meningitidis* in cerebrospinal fluid. *Frontiers in Microbiology*, 6, 1548.
- Lee, P. (2017). DNA amplification in the field: move over PCR, here comes LAMP. *Molecular Ecology Resources*, 17(2), 138–41.

- Liang, S., Chan, Y., Hsia, K., Lee, J., Kuo, M., & Hwa, K. (2009). Development of Loop-Mediated Isothermal Amplification Assay for Detection of *Entamoeba histolytica*. *Journal of Clinical Microbiology*, 1892–1895.
- Loftus, B., Anderson, I., Davies, R., Alsmark, U. C., Samuelson, J., & Amedeo, P. (2005). The genome of the protist parasite *Entamoeba histolytica*. *Nature*, 433(7028), 865-8
- Lorenzi, H. A., Puiu, D., Miller, J. R., Brinkac, L. M., Amedeo, P., Hall, N., ... & Caler, E. V. (2010). New Assembly, Reannotation and Analysis of the *Entamoeba histolytica* Genome Reveal New Genomic Features and Protein Content Information. *PLoS Neglected Tropical Diseases*, 4(6), e716.
- Markell, E. K., John, D. T., & Krotoski, W. A. (1999). *Lumen dwelling protozoa*. S Ozmot, Markell and Vogé's Medical Parasitology (8th ed.). Mexico: Saunders Company.
- Matey, E. J., Tokoro, M., Nagamoto, T., Mizuno, T., Saina, M. C., & Xiuqiong, B. (2016). Lower prevalence of *Entamoeba* species in children with vertically transmitted HIV infection in Western Kenya. *AIDS*, 30, 803-805.
- Mbae, C. K., Nokes, D. J., Mulinge, E., Nyambura, J., Waruru, A., & Kariuki, S. (2013). Intestinal parasitic infections in children presenting with diarrhoea in outpatient and inpatient settings in an informal settlement of Nairobi, Kenya. *BMC Infectious Diseases*, 13, 243.

- Mirelman, D., Nuchamowitz, Y., & Stolarsky, T. (1997). Comparison of use of enzyme-linked immunosorbent assaybased kits and PCR amplification of rRNA genes for simultaneous detection of *Entamoeba histolytica* and *E. dispar*. *Journal of Clinical Microbiology*, *35*, 2405–2407.
- Monis, P. T., Giglio, S., & Saint, C. P. (2005). Comparison of SYTO9 and SYBR Green I for real-time polymerase chain reaction and investigation of the effect of dye concentration on amplification and DNA melting curve analysis. *Analytical Biochemistry*, *340*, 24-34.
- Mori, Y., Nagamine, K., Tomita, N., & Notomi, T. (2001). Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochemical and Biophysics Research Communications*, *289*, 150-154.
- Mugambi, R. M., Agola, E. L., Mwangi, I. N., Kinyua, J., Shiraho, E. A., & Mkoji, G. M. (2015). Development and evaluation of a loop mediated isothermal amplification (LAMP) technique for the detection of hookworm (*Necator americanus*) infection in fecal samples. *Parasites and Vectors*, *8*, 574.
- Nagamine, K., Hase, T., & Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Molecular and Cellular Probes*, *16*, 223-229.

- Notomi, T., Mori, Y., Tomita, N., & Kanda, H. (2015). Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *Journal of Microbiology*, 53, 1-5.
- Njiru, Z. K. (2012). Loop-mediated isothermal amplification technology: towards point of care diagnostics. *PLoS Neglected Tropical Diseases*, 6(6), e1572.
- Njiru, Z. K., Mbae, C. K., & Mburugu, G. N. (2017). Loop-mediated isothermal amplification test for *Trypanosoma gambiense* group 1 with stem primers: a molecular xenomonitoring test for sleeping sickness. *Journal of Tropical Medicine*, 2017, 8630708
- Njiru, Z. K., Mikosza, A. S., Matovu, E., Enyaru, J. C., Ouma, J. O., & Kibona, S. N. (2008a). African trypanosomiasis: sensitive and rapid detection of the sub-genus *Trypanozoon* by loop-mediated isothermal amplification (LAMP) of parasite DNA. *International Journal of Parasitology*, 38, 589-99.
- Njiru, Z. K., Yeboah-Manu, D., Stinear, T. P., & Fyfe, J. A. (2012). Rapid and sensitive detection of *Mycobacterium ulcerans* by use of a loop-mediated isothermal amplification test. *Journal of Clinical Microbiology*, 50(5), 1737-1741.
- Notomi, T., Mori, Y., Tomita, N., & Kanda, H. (2015). Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *Journal of Microbiology*, 53, 1-5.

- Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, A. N., & Hase, T. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Research*, 28, E63.
- Nuñez, Y. O., Fernandez, M. A., Torres-Nunez, D., Silva, J. A., Montano, I., Maestre, J. L., & Fonte, L. (2001). Multiplex polymerase chain reaction amplification and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* DNA from stool samples. *American Journal of Tropical Medicine and Hygiene*, 64, 293–297.
- Nyarango, R. M., Aloo, P. A., Kabiru, E. W., & Nyanchongi B. O. (2008). The risk of pathogenic intestinal parasite infections in Kisii Municipality, Kenya. *BMC Public Health*, 8, 237.
- Pinheiro, S. M., Carneiro, R. M., Aca, I. S., Irmao, J. I., Morais, M. A. Jr., & Coimbra, M. R. (2004). Determination of the prevalence of *Entamoeba histolytica* and *E. dispar* in the Pernambuco state of northeastern Brazil by a polymerase chain reaction. *American Journal of Tropical Medicine and Hygiene*, 70, 221–224.
- Poon, L. L., Wong, B. W., Ma, E. H., Chan, K. H., Chow, L. M., Abeyewickreme, W., ... & Peiris, J. S. (2006). Sensitive and inexpensive molecular test for falciparum malaria: detecting *Plasmodium falciparum* DNA directly from heat-treated blood by loop-mediated isothermal amplification. *Clinical Chemistry*, 52, 303-306.

- Pritt, B. S., & Clark, C. G. (2008). Amoebiasis. *Mayo Clinic Proceedings*, 83, 1154-1159.
- Ramachandran, A., Bhattacharya, A., & Bhattacharya S. (1993). Nucleotide sequence analysis of the rRNA transcription unit of a pathogenic *Entamoeba histolytica* strain HM-1 :IMSSS. *Nucleic Acids Research*, 21, 8.
- Rao, S., Solaymani-Mohammadi, S., Petri, W. A. Jr., & Parker, S. K. (2009). Hepatic amebiasis: a reminder of the complications. *Current Opinion in Pediatrics*, 21, 145-149.
- Roy, S., Kabir, M., Mondal, D., Ali, I. K., Petri, W. A. Jr. & Haque, R. (2005). Real-time PCR assay for diagnosis of *Entamoeba histolytica* infection. *Clinical Microbiology*, 43, 2168–2172.
- Rivera, W. L., & Ong, V. A. (2013). Development of loop-mediated isothermal amplification for rapid detection of *Entamoeba histolytica*. *Asian Pacific Journal of Tropical Medicine*, 26(6), 457-61.
- Rivera, W. L., Tachibana, H., Silva-Tahat, M. R., Uemura, H., & Kanbara, H. (1996). Differentiation of *Entamoeba histolytica* and *E. dispar* DNA from cysts present in stool specimens by polymerase chain reaction: its field application in the Philippines. *Parasitology Research*, 82, 585–589.

- Saidi, S. M., Iijima, Y., Sang, W. K., Mwangudza, A. K., Oundo, J. O., & Taga, K. (1997). Epidemiological study on infectious diarrheal diseases in children in a coastal rural area of Kenya. *Microbiology and Immunology*, *41*, 773-778.
- Samie, A., Obi, L. C., Bessong, P. O., Stroup, S., Houpt, E., & Guerrant, R. L. (2006). Prevalence and species distribution of *E. histolytica* and *E. dispar* in the Venda region, Limpopo, South Africa. *American Journal of Tropical Medicine and Hygiene*, *75*, 565–571.
- Sargeant, P. G., Jackson, T. F., Wiffen, S., Bhojnani, R., Williams, J. E., Felmingham, D., ... & Phillips, E. (1987). The reliability of *Entamoeba histolytica* zymodemes in clinical laboratory diagnosis. *Archives of Investigative Medicine*, *18*, 69–75.
- Shiraho, E. A., Eric, A. L., Mwangi, I. N., Maina, G. M., Kinuthia, J. M., Mutuku, M. W., ... & Mkoji, G. M. (2016). Development of a loop mediated isothermal amplification for diagnosis of *Ascaris lumbricoides* in fecal samples. *Journal of Parasitology Research*, *2016*, 7376207.
- Stanley, S. L. Jr. (2003). Amoebiasis. *Lancet*, *361*(9362), 1025-34.
- Stauffer, W., Abd-Alla, M., & Ravdin, J. I. (2006). Prevalence and incidence of *Entamoeba histolytica* infection in South Africa and Egypt. *Archives of Medical Research*, *37*(2), 266-269.

- Sun, X. M., Ji, Y. S., Liu, X. Y., Xiang, M., He, G., Xie, L., ... & Suo, X. (2017). Improvement and evaluation of loop-mediated isothermal amplification for rapid detection of *Toxoplasma gondii* infection in human blood samples. *PLoS ONE*, *12*(1), e0169125.
- Tannich, E., & Burchard, G. D. (2010). Amoebiasis und andere Amöbeninfektionen. In: Löscher T, Burchard GD, editors. *Tropenmedizin in Klinik und Praxi*. Stuttgart: Thieme Verlag, 6, 45.
- Tanyuksel, M., & Petri, W. A. Jr. (2003). Laboratory diagnosis of amebiasis. *Clinical Microbiology Review*, *16*, 713–729.
- Tegege, B., Getie, S., Lemma, W., Mohon, A. N., & Pillai, D. R. (2017). Performance of loop mediated isothermal amplification (LAMP) for the diagnosis of malaria among malaria suspected pregnant women in Northwest Ethiopia. *Malaria Journal*, *16*(1), 34.
- Thekisoe, O. M., Bazie, R. S., Coronel-Servian, A. M., Sugimoto, C., & Kawazu, S. (2009). Stability of Loop-Mediated Isothermal Amplification (LAMP) reagents and its amplification efficiency on crude trypanosome DNA templates. *The Journal of Veterinary Medical Science*, *71*, 471–475.
- Verweij, J. J., Blotkamp, J., Brienen, E. A., Aguirre, A. & Polderman, A. M. (2000). Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* cysts using polymerase chain reaction on DNA isolated from faeces with spin columns.

European Journal of Clinical Microbiology & Infectious Diseases, 19, 358–361.

Viera, A. J., & Garrett, J. M. (2005). Understanding interobserver agreement: the kappa statistic. *Family Medicine*, 37(5), 360–3.

Visser, L. G., Verweij, J. J., Van Esbroeck, M., Edeling, W. M., Clerinx, J., & Polderman, A. M. (2006). Diagnostic methods for differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in carriers: performance and clinical implications in a non-endemic setting. *International Journal of Medical Microbiology*, 296, 397–403.

Walsh, J. A. (1986). Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. *Reviews of Infectious Diseases*, 8, 228–38.

Wang, L. T., Jen, G., & Cross, J. H. (1973). Establishment of *Entamoeba histolytica* from liver abscess in monoxenic cultures with hemoflagellates. *American Journal of Tropical Medicine and Hygiene*, 22, 30–32.

Wei, C., Wen-En, L., Yang-Ming, L., Shan, L., & Yi-Ming, Z. (2015). Diagnostic accuracy of loop-mediated isothermal amplification in detection of *Clostridium difficile* in stool samples: a meta-analysis. *Archives of Medical Science*, 11(5):927–36.

World Health Organization (1997). World Health Organization/Pan American Health Organization/UNESCO report of a consultation of experts on amoebiasis. *Weekly Epidemiology Records*, 72, 97–99.

World Health Organization. (2015). *WHO estimates of the global burden of foodborne diseases: Foodborne disease burden epidemiology reference group 2007-2015*. Geneva: WHO.

WHO/PAHO/UNESCO. (1997). A consultation with experts on amoebiasis. Mexico City, Mexico 28–29 January, 1997. *Epidemiological Bulletin*, 18, 13–4.

Ximénez, C., Cerritos, R., Rojas, L., Dolabella, S., Morán, P., & Shibayama, M. (2010). Human amebiasis: breaking the paradigm? *International Journal of Environmental Research and Public Health*, 7(3), 1105-20.

Zeyrek, F. Y., Turgay, N., Unver, A., Ustün, S., Akarca, U., & Töz S. (2013). Differentiation of *Entamoeba histolytica*/*Entamoeba dispar* by the polymerase chain reaction in stool samples of patients with gastrointestinal symptoms in the Sanliurfa Province. *Turkiye Parazitoloji Dergisi*, 37(3), 174-8.

APPENDICES

Appendix 1: Ethical approval


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 **August 29, 2014**

TO: **FRIDAH MWENDWA KIRIMI,**
PRINCIPAL INVESTIGATOR

THROUGH: **DR. WILLIE SANG,** *Forwarded*
ACTING DIRECTOR, CMR, *WJS 8/19/14*
NAIROBI

Dear Madam,

RE: SSC PROTOCOL NO. 2834 (RESUBMISSION): DEVELOPMENT AND EVALUATION OF A MODIFIED LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (mLAMP) TEST FOR THE DETECTION OF ENTAMOEBIA HISTOLYTICA (VERSION 1.1 DATED 25TH AUGUST 2014)

Reference is made to your letter dated 25th August, 2014. The ERC Secretariat acknowledges receipt of the revised protocol on August 26, 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the documents and is satisfied that the issues raised at the 229th meeting of the KEMRI ERC on 22nd July, 2014 have been adequately addressed.

The study is granted approval for implementation effective this **29th August, 2014**. Please note that authorization to conduct this study will automatically expire on **August 28, 2015**. 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 **July 17, 2015**.

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 SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,
EAB

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

In Search of Better Health

Appendix 2: Consent forms

APPENDIX B: CONSENT FORM ADULTS AGED 18 YEARS AND ABOVE

Title of the proposal: Differentiation and prevalence of pathogenic and non-pathogenic *Entamoeba spp* isolated from patients presenting with diarrhea at Mukuru-Reuben Clinic, Nairobi by nested multiplex polymerase chain reaction.

Principal Investigator: Erastus Mulinge Kakundi, **Co-Investigators:** Cecilia Mbae, Tabitha Irungu, Dr. Samuel Kariuki: P.O Box 19464-00202 Nairobi, Tel: 020-2722541 Ext 3339, or 0722 935320

You are being invited to take part in this research study because you presented with diarrhea, abdominal pain or discomfort. Diarrhea is a serious disease in young children and adults as well. *Entamoeba histolytica* is one of the germs that causes diarrhea, abdominal pain and discomfort, which you can get from contaminated food or water. The purpose of the study is to find out if you are infected with this disease causing organism or not. The results of this study will help us determine the accurate cause of diarrhea to enable improve treatment. The study will involve everyone older than 5 years presenting to the clinic with diarrhea, abdominal pain or discomfort and have agreed to participate in the study and signed this consent form. In order to carry out laboratory tests, we will require obtaining a stool sample from you. The stool specimen will be stored at the Centre for Microbiology Research, KEMRI. **With the approval of the study subjects isolates with unique characteristics may require full genome sequencing, which will be done at Wellcome Trust Sanger Institute, United Kingdom.**

Risks and benefits

Stool specimen collection is a routine medical procedure, non-invasive and therefore painless. Results from the stool analysis will be made available to the clinicians managing you for appropriate action.

Participant's rights

If you have read this form and have decided to participate in this project, please understand your participation is voluntary and you have the right to withdraw your consent or discontinue participation at any time without penalty. **Additionally, you are free to accept or decline your specimen being examined at Wellcome Trust Sanger Institute, UK in the event that unique isolates are detected from it.** Your individual privacy will be maintained in all published and written data resulting from the study.

If you have questions about your rights as a study participant, or you are dissatisfied at any time with any aspect of this study, you may contact the investigators on the contacts given above or The Secretary KEMRI ERC office. P.O Box 54840-00200, Nairobi Tel. 2722541 Ext. 3307. I have read this form or had it read to me. I have discussed the information with clinician. My questions have been answered. My decision whether or not to take part in the study is voluntary.

Participant's Name Signature Date

Clinician's Name Signature Date

Witness's Name Signature Date

KIAMBATISHO C: FOMU YA IDHINI YA WATU WA UMRI WA MIAKA KUMI NA NANE NA ZAIDI

Kichwa cha pendekezo: Tofauti na kiwango cha maambukizi ya *Entamoeba spp* inayodhuru kutoka kwa isiyo dhuru katika wagonjwa wanaotembela Mukuru-Reuben Kliniki, Nairobi na mauvumivu ya kuhara kwa njia ya Furushi mmenyuko multiplex polimeresi.

Mpelelezi Mkuu: Erastus Mulinge Kakundi; **Wapelelezi wenzangu:** Cecilia Mbae, Tabitha Irungu, Dr Samuel Kariuki: PO Box 19464-00202 Nairobi, Tel: 020-2722541 ext 3339, au 0722 935320.

Unaalikwa kushiriki katika utafiti huu, kwa sababu una maumivu au usumbufu wa tumbo na kuhara. Kuharisha ni ugonjwa mbaya kwa watoto wadogo na watu wazima pia. *Entamoeba histolytica* ni moja ya wadudu wanaosababisha kuharisha, maumivu na usumbufu wa tumbo ambao unaweza kupata kutoka kwa chakula au maji machafu. Madhumuni ya utafiti huu, ni kujua kama umeambukizwa ugonjwa unao sababisha viumbe hivi au la. Matokeo ya utafiti huu yataonyesha sababu sahihi ya kuhara ili kuboresha matibabu. Utafiti unahusisha kila mtu wa umri zaidi ya miaka 5 wanaotembelea Mukuru-Reuben kliniki na maumivu au usumbufu wa tumbo na kuhara na wamekubali kushiriki katika utafiti na kutia sahihi fomu za ridhaa. Ili kufanya vipimo vya maabara, itahitajika kupata sampuli ya kinyesi chako. Specimen ya kinyesi itahifadhiwa katika Kituo cha Utafiti cha Mikrobiolojia, KEMRI. **Sampuli zenye sifa ya kipekee zitahitaji full genome sequencing, ambayo itafanyika katika Taasisi ya Wellcome Trust Sanger, Uingereza, kufuatia idhini ya mshiriki.**

Hatari na faida

Ukusanyaji wa specimen ya kinyesi ni utaratibu wa kawaida wa matibabu, mashirika yasiyo vamizi na hivyo hakuna uchungu. Matokeo ya uchambuzi wa kinyesi, yatapatikana kwa daktari kwa ajili ya hatua sahihi.

Haki za mshiriki

Kama umesoma fomu hii na umeamua kushiriki katika utafiti huu, tafadhali inafaa kuelewa ushiriki wako ni wa hiari na una haki ya kutoa idhini yako au kusimamisha ushiriki wakati wowote bila adhabu. **Zaidi ya hayo, wewe ni una uhuru wa kukubali**

au kukataa specimen yako ya kukaguliwa katika Taasisi ya Wellcome Trust Sanger, Uingereza kama sampuli itapatikana na sifa za kipekee. Siri yako binafsi itaifadhiwa katika data zote zitakaozochapishwa na kuandikwa kutokana na utafiti huu. Kama una maswali kuhusu haki zako kama mshiriki katika utafiti huu, au hujaridhika wakati wowote na dhana yoyote ya utafiti huu, unaweza kuwasiliana na wachunguzi kwa mawasiliano yenye kupeanwa hapo juu ama kwa katibu mkuu, KEMRI ERC PO Box 54840-00200, Nairobi Tel. 2722541 ext. 3307. Nimesoma fomu hii au nimesomewa. Mimi nimejadili taarifa na daktari. Maswali yangu yamejibiwa. Uamuzi wangu kushiriki katika utafiti ni kwa hiari.

Jina la mshiriki.....	Sahihi.....	Tarehe.....
Jina la daktari -----	Sahihi	Tarehe
Jina la shahidi	Sahihi	Tarehe

APPENDIX D: CONSENT FORM CHILDREN AGED 5 – 18 YEARS

Title of the proposal: Differentiation and prevalence of pathogenic and non-pathogenic *Entamoeba spp* isolated from patients presenting with diarrhea at Mukuru-Reuben Clinic, Nairobi by nested multiplex polymerase chain reaction.

Principal Investigator: Erastus Mulinge Kakundi, **Co-Investigators:** Cecilia Mbae, Tabitha Irungu, Dr. Samuel Kariuki: P.O Box 19464-00202 Nairobi, Tel: 020-2722541 Ext 3339, or 0722 935320

Your child is being invited to take part in this research study because has presented with diarrhea, abdominal pain or discomfort. Diarrhea is a serious disease in young children and adults as well. *Entamoeba histolytica* is one of the germs is that causes diarrhea, abdominal pain and discomfort, which you can get from contaminated food or water. The purpose of the study is to find out if your child is infected with this disease causing organism or not. The results of this study will help us determine the accurate cause of diarrhea to enable improve treatment. The study will involve everyone older than 5 years presenting to the clinic with diarrhea, abdominal pain or discomfort and their parent/guardian has allowed the child to participate in the study and signed this consent form. In order to carry out laboratory tests, we will require obtaining a stool sample from your child. The stool specimen will be stored at the Centre for Microbiology Research, KEMRI. **With the approval of the study subjects isolates with unique characteristics may require full genome sequencing, which will be done at Wellcome Trust Sanger Institute, United Kingdom.**

Risks and benefits

Stool specimen collection is a routine medical procedure, non-invasive and therefore painless. Results from the stool analysis will be made available to the clinicians managing your child for appropriate action.

Participant's rights

If you have read this form and have decided to allow your child to participate in this project, please understand his/her participation is voluntary and you have the right to

withdraw your consent or discontinue his/her participation at any time without penalty. **Additionally, you are free to accept or decline your child specimen being examined at Wellcome Trust Sanger Institute, UK in the event of unique isolates are detected from it.** The child's individual privacy will be maintained in all published and written data resulting from the study.

If you have questions about the child's rights as a study participant, or you are dissatisfied at any time with any aspect of this study, you may contact the investigators on the contacts given above or The Secretary KEMRI ERC office. P.O Box 54840-00200, Nairobi Tel. 2722541 Ext. 3307. I have read this form or had it read to me. I have discussed the information with clinician. My questions have been answered. The decision whether or not my child to take part in the study allow is voluntary.

Parents/Guardian Name Signature Date

Clinician's Name Signature Date

Witness's Name Signature Date

KIAMBATISHO E: FOMU YA IDHINI UMRI WA MIAKA 5 – 18

Kichwa cha pendekezo: Tofauti na kiwango cha maambukizi ya *Entamoeba spp* inayodhuru kutoka kwa isiyo dhuru katika wagonjwa wanaotembela Mukuru-Reuben Klini, Nairobi na mauvumivu ya kuhara kwa njia ya Furushi mmenyuko multiplex polimeresi.

Mpelelezi Mkuu: Erastus Mulinge Kakundi; **Wapelelezi wezangu:** Cecilia Mbae, Tabitha Irungu, Dr Samuel Kariuki: PO Box 19464-00202 Nairobi, Tel: 020-2722541 ext 3339, au 0722 935320.

Mtoto wako anaalikwa kushiriki katika utafiti huu, kwa sababu ana maumivu au usumbufu wa tumbo na kuhara. Kuharisha ni ugonjwa mbaya kwa watoto wadogo na watu wazima pia. *Entamoeba histolytica* ni moja ya wadudu wanaosababisha kuharisha, maumivu au usumbufu wa, ambao unaweza kupata kutoka kwa chakula au maji machafu. Madhumuni ya utafiti huu, ni kujua kama ameambukizwa ugonjwa hunao sababishwa na viumbe hivi au la. Matokeo ya utafiti huu yataonyesha sababu sahihi ya kuhara ili kuboresha matibabu. Utafiti unahusisha kila mtu umri wa zaidi ya miaka 5 wanotembelea Mukuru-Reuben klini na maumivu au usumbufu wa tumbo na kuhara na mzazi/mlezi amekubali mtoto wake kushiriki katika utafiti na kutia sahihi fomu za ridhaa. Ili kufanya vipimo vya maabara, itahitajika kupata sampuli ya kinyesi cha mtoto wako. Specimen ya kinyesi itahifadhiwa katika Kituo cha Utafiti cha Mikrobiolojia, KEMRI. **Sampuli zenye sifa ya kipekee zitahitaji *full genome sequencing*, ambayo itafanyika katika Taasisi ya Wellcome Trust Sanger, Uingereza, kufuatia idhini yako kwa niaba ya mtoto wako.**

Hatari na faida

Ukusanyaji wa specimen ya kinyesi ni utaratibu wa kawaida wa matibabu, mashirika yasiyo vamizi na hivyo hakuna uchungu. Matokeo ya uchambuzi wa kinyesi, yatapatikana kwa daktari kwa ajili ya hatua sahihi.

Haki za mshiriki

Kama umesoma fomu hii na umekubali mtoto wako kushiriki katika utafiti huu, tafadhali inafaa kuelewa ushiriki wake ni kwa hiari na una haki ya kutoa idhini yake au kusimamisha ushiriki wake wakati wowote bila adhabu. **Zaidi ya hayo, wewe ni huru**

kukubali au kukataa specimen yake kukaguliwa katika Taasisi ya Wellcome Trust Sanger, Uingereza kama sampli itapatikana na sifa za kipekee. Siri ya mtoto wako binafsi itaifadhiwa katika data zote zitakazochapishwa na kuandikwa kutokana na utafiti huu. Kama una maswali kuhusu haki za mtoto wako kama mshiriki katika utafiti huu, au hujaridhika wakati wowote na dhana yoyote ya utafiti huu, unaweza kuwasiliana na wachunguzi kwa mawasiliano yenye yamepeanwa hapo juu ama kwa katibu mkuu, KEMRI ERC PO Box 54840-00200, Nairobi Tel. 2722541 ext. 3307. Nimesoma fomuu hii au nimesomewa. Mimi nimejadili taarifa na daktari. Maswali yangu yamejibiwa. Uamuzi wangu kuruhusu mtoto wangu kushiriki katika utafiti ni kwa hiari.

Jina la mzazi/mlezi	Sahihi.....	Tarehe.....
Jina la daktari	Sahihi	Tarehe
Jina la shahidi	Sahihi	Tarehe

**APPENDIX F: PARENTAL PERMISSION FOR PARTICIPANTS AGED
12-17 YEARS OLD**

Title of the proposal: Differentiation and prevalence of pathogenic and non-pathogenic *Entamoeba spp* isolated from patients presenting with diarrhea at Mukuru-Reuben Clinic, Nairobi by nested multiplex polymerase chain reaction.

Principal Investigator: Erastus Mulinge Kakundi, **Co-Investigators:** Cecilia Mbae, Tabitha Irungu, Dr. Samuel Kariuki: P.O Box 19464-00202 Nairobi, Tel: 020-2722541 Ext 3339, or 0722 935320.

I give permission/authorize my child to participate in this study. I have read the consent form, and the information therein has been explained to me clearly. All my questions have been fully answered and I understand that my decision whether or not allow my child to take part in the study is voluntary.

Parents/Guardian Name Signature Date

Clinician's Name Signature Date

Witness's Name Signature Date

KIAMBATISHO G: RUHUSA YA WAZAZI KWA WASHIRIKI WENYE UMRI WA MIAKA 12-17

Kichwa cha pendekezo: Tofauti na kiwango cha maambukizi ya *Entamoeba spp* inayodhuru kutoka kwa isiyodhuru katika wagonjwa wanaotembela Mukuru-Reuben Klini, Nairobi na mauvumivu ya kuhara kwa njia ya Furushi mmenyuko multiplex polimeresi.

Mpelelezi Mkuu: Erastus Mulinge Kakundi; **Wapelelezi wezangu:** Cecilia Mbae, Tabitha Irungu, Dr Samuel Kariuki: PO Box 19464-00202 Nairobi, Tel: 020-2722541 ext 3339, au 0722 935320.

Mimi nimetoa idhini ya mtoto wangu kushiriki katika utafiti huu. Nimesoma fomu ya ridhaa na habari ndani yake imeelezwa kikamilifu. Maswali yangu yote yamejibiwa vizuri na mimi naelewa kwamba uamuzi wangu kama kuruhusu au kutoruhusu mtoto wangu kushiriki katika utafiti ni kwa hiari.

Jina la mzazi/mlezi	Sahihi.....	Tarehe.....
Jina la daktari	Sahihi	Tarehe
Jina la shahidi	Sahihi	Tarehe

APPENDIX H: ASSENT FOR PARTICIPANTS AGED 12 – 17 YEARS

Title of the proposal: Differentiation and prevalence of pathogenic and non-pathogenic *Entamoeba spp* isolated from patients presenting with diarrhea at Mukuru-Reuben Clinic, Nairobi by nested multiplex polymerase chain reaction.

Principal Investigator: Erastus Mulinge Kakundi, **Co-Investigators:** Cecilia Mbae, Tabitha Irungu, Dr. Samuel Kariuki: P.O Box 19464-00202 Nairobi, Tel: 020-2722541 Ext 3339, or 0722 935320.

I with the permission of my parents/guardian, confirm that i will participate in this study. I have read and understood the information in the consent form, and all the details of the study have been explained to me. All my questions have been answered and I do understand that my decision whether or not to take part in the study is voluntary.

Participant’s Name Signature Date

Clinician’s Name Signature Date

Witness’s Name Signature Date

KIAMBATISHO I: KUTIWA SAINI KWA AJILI YA WASHIRIKI WENYE UMRI WA MIAKA 12-17

Kichwa cha pendekezo: Tofauti na kiwango cha maambukizi ya *Entamoeba spp* inayodhuru kutoka kwa isiyo dhuru katika wagonjwa wanaotembela Mukuru-Reuben Kliniki, Nairobi na mauvumivu ya kuhara kwa njia ya Furushi mmenyuko multiplex polimeresi.

Mpelelezi Mkuu: Erastus Mulinge Kakundi; **Wapelelezi wezangu:** Cecilia Mbae, Tabitha Irungu, Dr Samuel Kariuki: PO Box 19,464-00,202 Nairobi, Tel: 020-2722541 ext 3339, au 0722 935320.

Mimi kwa idhini ya wazazi wangu / mlezi, nadhibitisha kwamba nitashiriki katika utafiti huu. Nimeisoma na nimeielewa habari iliyoko katika fomu ya ridhaa, na nimepewa maelezo ya utafiti huu. Maswali yangu yote yamejibiwa na niaelewa kwamba uamuzi wangu kama kushiriki au kutoshiriki katika utafiti ni kwa hiari.

Jina la mshiriki.....	Sahihi.....	Tarehe.....
Jina la daktari -----	Sahihi	Tarehe
Jina la shahidi	Sahihi	Tarehe

**APPENDIX J: CONSENT/ASSENT FOR STORAGE AND SHIPMENT OF
ENTAMOEBEA SPP ISOLATES**

Title of the proposal: Differentiation and prevalence of pathogenic and non-pathogenic *Entamoeba spp* isolated from patients presenting with diarrhea at Mukuru-Reuben Clinic, Nairobi by nested multiplex polymerase chain reaction.

Principal Investigator: Erastus Mulinge Kakundi, **Co-Investigators:** Cecilia Mbae, Tabitha Irungu, Dr. Samuel Kariuki: P.O Box 19464-00202 Nairobi, Tel: 020-2722541 Ext 3339, or 0722 935320.

I have given permission for my/ my child's stool specimen to be stored at Centre for Microbiology Research, KEMRI, Parasitology laboratory for upto five years after the completion of the study, in case there is need to refer back to the stool specimen for any further information. I also agree that my/ my child's stool specimen can be shipped out of the country for further analysis if need be.

Parents/Guardian Name Signature Date
Clinician's Name Signature Date
Witness's Name Signature Date

**KIAMBATISHO K: RIDHAA/ KUTIWA SAINI KWA AJILI YA KUHIFADHI
NA USAFIRISHAJI WA ENTAMOEBA SPP HUTENGA**

Kichwa cha pendekezo: Tofauti na kiwango cha maambukizi ya *Entamoeba spp* inayodhuru kutoka kwa isiyo dhuru katika wagonjwa wanaotembela Mukuru-Reuben Kliniki, Nairobi na mauvumivu ya kuhara kwa njia ya Furushi mmenyuko multiplex polimeresi.

Mpelelezi Mkuu: Erastus Mulinge Kakundi; **Wapelelezi wezangu:** Cecilia Mbae, Tabitha Irungu, Dr Samuel Kariuki: PO Box 19,464-00,202 Nairobi, Tel: 020-2722541 ext 3339, au 0722 935320.

Mimi nimekubali kinyesi changu/cha mwanangu kuhifadhiwa katika Kituo cha Utafiti wa Microbiologia, KEMRI, maabara ya parasitologia, kwa muda usiozidi miaka mitano baada ya kukamilika kwa utafiti huu, katika kesi kuna haja ya kurejea kwa hizi specimeni kwa maelezo zaidi. Mimi pia nimekubali kwamba kinyesi changu / cha mtoto wangu kinaweza kusafirishwa nje ya nchi kwa ajili ya uchambuzi zaidi kama kutakuwa na haja.

Jina la mzazi/mlezi/mtoto Sahihi..... Tarehe.....

Jina la daktari Sahihi Tarehe

Jina la shahidi Sahihi Tarehe

Appendix 3: Protocol - Isolation of DNA from Stool for Pathogen Detection

Procedure

1. Weigh 180–220 mg stool in a 2 ml microcentrifuge tube (not provided) and place the tube on ice. This protocol is optimized for use with 180–220 mg stool but can also be used with smaller amounts. There is no need to reduce the amounts of buffers or InhibitEX matrix when using smaller amounts of stool. For samples >220 mg, see “Protocol: Isolation of DNA from Larger Volumes of Stool”, page 30. If the sample is liquid, pipet 200 µl into the microcentrifuge tube. Cut the end of the pipet tip to make pipetting easier. If the sample is frozen, use a scalpel or spatula to scrape bits of stool into a 2 ml microcentrifuge tube on ice. Note: When using frozen stool samples, take care that the samples do not thaw until Buffer ASL is added in step 2 to lyse the sample; otherwise the DNA in the sample may degrade. After addition of Buffer ASL, all following steps can be performed at room temperature (15–25°C).

2. Add 1.4 ml Buffer ASL to each stool sample. Vortex continuously for 1 min or until the stool sample is thoroughly homogenized. Note: It is important to vortex the samples thoroughly. This helps ensure maximum DNA concentration in the final elute.

3. Heat the suspension for 5 min at 70°C. This heating step increases total DNA yield 3- to 5-fold and helps to lyse bacteria and other parasites. The lysis temperature can be increased to 95°C for cells that are difficult to lyse (such as Gram-positive bacteria).

4. Vortex for 15 s and centrifuge sample at full speed for 1 min to pellet stool particles.

5. Pipet 1.2 ml of the supernatant into a new 2 ml microcentrifuge tube (not provided) and discard the pellet. Note: The 2 ml tubes used should be wide enough to accommodate an InhibitEX Tablet. Transfer of small quantities of pelleted material will not affect the procedure.

6. Add 1 InhibitEX Tablet to each sample and vortex immediately and continuously for 1 min or until the tablet is completely suspended. Incubate suspension for 1 min at room temperature to allow inhibitors to adsorb to the InhibitEX matrix.

7. Centrifuge sample at full speed for 3 min to pellet inhibitors bound to InhibitEX matrix. Stool Pathogen Detection Stool Pathogen Detection 16 QIAamp DNA Stool Handbook 06/2012

8. Pipet all the supernatant into a new 1.5 ml microcentrifuge tube (not provided) and discard the pellet. Centrifuge the sample at full speed for 3 min. Transfer of small quantities of pelleted material from step 7 will not affect the procedure.

9. Pipet 15 µl proteinase K into a new 1.5 ml microcentrifuge tube (not provided).

10. Pipet 200 µl supernatant from step 8 into the 1.5 ml microcentrifuge tube containing proteinase K.

11. Add 200 µl Buffer AL and vortex for 15 s. Note: Do not add proteinase K directly to Buffer AL. It is essential that the sample and Buffer AL are thoroughly mixed to form a homogeneous solution.

12. Incubate at 70°C for 10 min. Centrifuge briefly to remove drops from the inside of the tube lid (optional).

13. Add 200 μ l of ethanol (96–100%) to the lysate, and mix by vortexing. Centrifuge briefly to remove drops from the inside of the tube lid (optional).

14. Label the lid of a new QIAamp spin column placed in a 2 ml collection tube. Carefully apply the complete lysate from step 13 to the QIAamp spin column without moistening the rim. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the tube containing the filtrate. Close each spin column to avoid aerosol formation during centrifugation. If the lysate has not completely passed through the column after centrifugation, centrifuge again until the QIAamp spin column is empty.

15. Carefully open the QIAamp spin column and add 500 μ l Buffer AW1. Close the cap and centrifuge at full speed for 1 min. Place the QIAamp spin column in a new 2 ml collection tube, and discard the collection tube containing the filtrate.

16. Carefully open the QIAamp spin column and add 500 μ l Buffer AW2. Close the cap and centrifuge at full speed for 3 min. Discard the collection tube containing the filtrate. Note: Residual Buffer AW2 in the elute may cause problems in downstream applications. Some centrifuge rotors may vibrate upon deceleration, resulting in the flow-through, which contains Buffer AW2, contacting the QIAamp spin column. Removing the QIAamp spin column and collection tube from the rotor may also cause flow-through to come into contact with the QIAamp spin column.

17. Recommended: Place the QIAamp spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for

1 min. This step helps to eliminate the chance of possible Buffer AW2 carryover. Stool

Pathogen Detection

18. Transfer the QIAamp spin column into a new, labeled 1.5 ml microcentrifuge tube (not provided). Carefully open the QIAamp spin column and pipet 200 μ l Buffer AE directly onto the QIAamp membrane. Close the cap and incubate for 1 min at room temperature, then centrifuge at full speed for 1 min to elute DNA. Note: When using elutes in PCR, for maximum PCR robustness we highly recommend adding BSA to a final concentration of 0.1 μ g/ μ l to the PCR mixture. For maximum PCR specificity we recommend using HotStarTaq Plus DNA Polymerase (see ordering information on page 38). For best results in downstream PCR, use the minimum amount of elute possible in PCR; the volume of elute used as template should not exceed 10% of the final volume of the PCR mixture. Also, note that high amounts of template DNA may inhibit the PCR. DNA yield is typically 15–60 μ g but, depending on the individual stool sample and the way it was stored, may range from 5 to 100 μ g. DNA concentration is typically 75–300 ng/ μ l.

Appendix 4: Protocol for stem 18S LAMP test for *Entamoeba histolytica*

Sample preparation from stool

1. For the liquid stool sample, pipette 200 µl into a 2 ml microcentrifuge tube. If the sample is solid, weigh 200 mg in a 2 ml microcentrifuge tube.
2. Wash the stool sample five times using distilled water. Add 500 µl distilled water in the microcentrifuge tube containing the stool sample. Vortex for 15 seconds then centrifuge for one minute at full speed. Discard the supernatant. Repeat five times.
3. After the last wash, add 1.4 ml of lysis buffer and subject to five times 30-minutes thawing (80°C) and freezing (-80°C) to rupture the rigid cysts.
4. Vortex for 15 s and centrifuge sample at full speed for 1 min to pellet stool particles.
5. Pipette 1.2 ml of the supernatant to prepare the DNA. Follow manufacturer's instructions on the selected kit for isolation of DNA from stool.
6. Elute the genomic DNA in 50 µl nuclease-free water and store at -20°C until use.

Primers and dNTPs dilution

1. Dilute the primers as follows using nuclease-free water: FIP/BIP to final concentration of 35 pmol each, LF/LB to 18 pmol each and SF/SB so as to have a final concentration of 23 pmol.
2. Dilute dNTPs to have a final concentration of 2 mM.

Preparation of master mix

1. A final 25 μL reaction is composed of 2.5 μL 10x buffer, 1 μL 2 mM dNTPs, 1 μL each primer set, 1 μL of 1M betaine, 0.5 μL 8000 U/ml *Bst* Polymersae and 12 μL nuclease-free water.

Addition of template

1. To the master mix, add 1-2 μL of the template (sample) to have a final volume of 25 μL .
2. Include negative and positive controls.

LAMP reaction

1. Adjust temperature on a heating block or water bath to 62°C.
2. Run the LAMP reactions for 50 minutes.

Results reading

1. Add 1/10 μL dilution of SYBR® Green 1 dye to the reaction tubes
2. A positive sample will fluoresce green and a negative sample will remain orange.

Appendix 5: Publication

Mwendwa et al. *BMC Res Notes* (2017) 10:142
DOI 10.1186/s13104-017-2466-3

BMC Research Notes

RESEARCH ARTICLE

Open Access



Stem loop-mediated isothermal amplification test: comparative analysis with classical LAMP and PCR in detection of *Entamoeba histolytica* in Kenya

Fridah Mwendwa^{1*}, Cecilia K. Mbae², Johnson Kinyua³, Erastus Mulinge², Gitonga Nkanata Mburugu⁴ and Zablon K. Njiru^{4,5}

Abstract

Background: *Entamoeba histolytica*, the causative agent for amoebiasis is a considerable burden to population in the developing countries where it accounts for over 50 million infections. The tools for detection of amoebiasis are inadequate and diagnosis relies on microscopy which means a significant percent of cases remain undiagnosed. Moreover, tests formats that can be rapidly applied in rural endemic areas are not available.

Methods: In this study, a loop-mediated isothermal test (LAMP) based on 18S small subunit ribosomal RNA gene was designed with extra reaction accelerating primers (stem primers) and compared with the published LAMP and PCR tests in detection of *E. histolytica* DNA in clinical samples.

Results: The stem LAMP test indicated shorter time to results by an average 11 min and analytical sensitivity of 10^{-7} (~30 pg/ml) compared to the standard LAMP and PCR which showed sensitivities levels of 10^{-5} (~3 ng/ml) and 10^{-4} (~30 ng/ml) respectively using tenfold serial dilution of DNA. In the analysis of clinical specimens positive for *Entamoeba* spp. trophozoites and cysts using microscopy, the stem LAMP test detected *E. histolytica* DNA in 36/126, standard LAMP test 20/126 and PCR 17/126 cases respectively. There was 100% agreement in detection of the stem LAMP test product using fluorescence of SYTO-9 dye in real time machine, through addition of 1/10 dilution of SYBR[®] Green I and electrophoresis in 2% agarose gel stained with ethidium bromide.

Conclusion: The stem LAMP test developed in this study indicates potential towards detection of *E. histolytica*.

Keywords: Amoebiasis, *Entamoeba histolytica*, Diagnosis, Loop-mediated isothermal amplification, LAMP, Stem-LAMP test, Kenya

Background

Amoebiasis caused by protozoan *Entamoeba histolytica* is an important human gastrointestinal infection responsible for over 50 million amoebic infection cases with over 100,000 deaths annually [1]. It is a leading cause of death only surpassed by malaria and schistosomiasis [2]

with most of these cases being reported in the developing countries [3–5]. In Africa, the burden of amoebiasis is high with an estimated *E. histolytica* infection median rate of 796 per 100,000 people [6]. Studies conducted in Kenya indicated prevalence of 6–11% of *E. histolytica/Entamoeba dispar* in children at selected hospitals [4, 5, 7] and 11–32% among adults [8]. Moreover, in more recent studies, the prevalence of *E. histolytica* by qPCR was recorded at 15% in Bungoma County, Western Kenya [9] while a much lower prevalence of 0.4% was reported among children with vertically transmitted HIV infection [10]. This data suggests that amoebiasis is

*Correspondence: fridahmwendwa.fm@gmail.com

¹ Institute of Tropical Medicine and Infectious Diseases, Jomo Kenyatta University of Agriculture and Technology, PO Box 62000-0200, Nairobi, Kenya

Full list of author information is available at the end of the article



© The Author(s) 2017. This article is distributed under the terms of the Creative Commons Attribution 4.0 International License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The Creative Commons Public Domain Dedication waiver (<http://creativecommons.org/publicdomain/zero/1.0/>) applies to the data made available in this article, unless otherwise stated.