EVALUATION OF LOOP-MEDIATED ISOTHERMAL AMPLIFICATION AS A 
POINT-OF-CARE DIAGNOSTIC TOOL FOR MYCOBACTERIUM TUBERCULOSIS

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A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT FOR THE AWARD OF 
THE MASTER OF SCIENCE DEGREE IN MEDICAL MICROBIOLOGY AT THE 
UNIVERSITY OF NAIROBI
DECLARATION

I declare that this dissertation is my original work and has not been presented for a degree or diploma in any other university.

Sylvia Wambui Thion’o

H56/68369/2011

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Signed……………………………………………………………..Date……………………………………
DEDICATION

I dedicate this project to my parents, John Thiong’o Mwangi and the Late Agnes Muthoni Mwangi, for their immense support and constant encouragement.
ACKNOWLEDGEMENTS

I would like to thank the Almighty God for his grace and faithfulness while pursuing this project.

I would also like to thank my supervisors Prof. Omu Anzala and Dr. Julius Oyugi, for their guidance and support in writing this thesis.

I would like to thank the National Tuberculosis Reference Laboratory (NTRL) for their assistance during sample collection, processing and analysis.

I want to acknowledge the KNH/UON Ethics and Research Committee for approving this project.

I also want to acknowledge my friends and family for their support and prayers.

Last but not least, I want to acknowledge my colleagues for their encouragement and support during this study.
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ABBREVIATIONS

AFB- Acid-fast bacilli
BSC- Biosafety cabinet
DNA- Deoxyribonucleic acid
dNTPs- deoxynucleotide triphosphates
DST- Drug susceptibility testing
ELISA- Enzyme-linked immunosorbent assay
ELISPOT- Enzyme-linked immunospot
FP – False positive
HIV- Human immunodeficiency virus
IGRA- Interferon gamma release assays
Ig- Immunoglobulin
IFN-γ- Interferon gamma
LAMP- Loop-mediated isothermal amplification
LJ- Lowenstein-Jensen
LTBI- Latent tuberculosis infection
MGIT- Mycobacteria growth indicator tube
MODS- Microscopic-observed drug susceptibility
NAAT- Nucleic acid amplification tests
NALC- N-acetyl-L-cysteine
NaOH- Sodium hydroxide
NPV- Negative predictive value

NTM- Non-tuberculous Mycobacteria

NTRL- National Tuberculosis Reference Laboratory

PBMC- Peripheral blood mononuclear cell

PCR- Polymerase chain reaction

PPD- Purified protein derivative

PPV- Positive predictive value

TB- Tuberculosis

TP- True positive

UNITID- University of Nairobi Institute of Tropical and Infectious Diseases

UV- Ultra-violet

WHO- World Health Organization

ZN- Ziehl Neelsen
ABSTRACT

Background: The bacterium *Mycobacterium tuberculosis* is the causative agent of tuberculosis. According to the World Health Organization (WHO), there were an estimated 8.8 million incident cases of tuberculosis globally in 2010. Kenya is on the list of 22 high-burden TB countries in the world and has the fifth highest burden in Africa. The loop-mediated isothermal amplification (LAMP) technique has the potential to serve as a simple, rapid, specific and cost-effective point-of-care diagnostic tool.

Broad Objective: To evaluate the performance of LAMP against culture on Lowenstein-Jensen for diagnosis of tuberculosis.

Study Design: This study was a cross-sectional study. LAMP assay was evaluated against culture on LJ for the diagnosis of tuberculosis.

Methodology: Culture on LJ medium was conducted at the National Tuberculosis Reference Laboratory. LAMP assay was conducted at UNITID. A set of 6 primers, comprising 2 outer primers (F3 and B3), 2 inner primers (FIP and BIP), and 2 loop primers (FLP and BLP), were used. LAMP amplicons in the reaction tube were detected under UV light after adding 1.0 μl of 1/10-diluted original SYBR Green I to the tube and observed the color of the solution. The solution fluoresced green in the presence of a LAMP amplicon, while it remained orange with no amplification.

Results: The study showed a sensitivity of 98.6%. This means that 98.6% of the study population was declared to be diseased and 1.4% of the population was falsely declared to be disease-free by the test. The study showed a specificity of 96.4%, which means that 96.4% of the study population was declared to be disease-free, while 3.6% was falsely declared to be diseased by LAMP.

Conclusion: Due to its ease of use in developing countries and its high sensitivity and specificity, this assay may be adopted to facilitate the identification of *M. tuberculosis* without the use of culture.
CHAPTER 1

1.1. BACKGROUND

The bacterium *Mycobacterium tuberculosis* is the causative agent of tuberculosis (TB). TB has plagued humankind worldwide for thousands of years. Despite the first anti-tuberculosis drugs being discovered more than 60 years ago, TB today still kills an estimated 1.7 million people each year [Lawn and Zumla, 2011].

From the time of Robert Koch’s identification of the causative agent of TB until relatively recently, progress had been relatively slow in understanding the basic biology of *M. tuberculosis* [Schluger. 2005]. The organism is an obligate intracellular pathogen that can infect several animal species, although human beings are the principal hosts. It is an aerobic, acid-fast, non-motile, non-encapsulated, non-spore forming bacillus. It grows most successfully in tissues with high oxygen content, such as the lungs [Lawn and Zumla, 2011].

*M. tuberculosis* has no known reservoir outside man, and the environments to which the organism must respond are defined solely by its natural history within its host. TB is a disease that is almost exclusively transmitted by aerosolized droplets containing infectious *M. tuberculosis*. These inhaled bacilli lodge in the terminal air spaces of the lung where they enter and replicate within alveolar macrophages. Although the human immune response is highly effective in controlling the primary infection, the organism is almost never eradicated [Glickman and Jacobs, 2001].

In Kenya, TB culture is reserved for the evaluation of all pulmonary TB patients who have failed initial or re-treatment, relapsed or are returning to treatment after a period of default because these patients may have drug resistant TB bacilli. [Sitienei *et al.*,w Division of Leprosy, TB and Lung Disease]. Apart from the reference laboratory, Moi Teaching and Referral Hospital, Homabay (MSF), Nairobi Hospital-Private, Agha Khan Hospital-Private and KEMRI/CRDR, mainly research, are the only other facilities equipped to carry out cultures [http://www.nltp.co.ke/index.php?option=com_content&view=article&id=14:tbcrl&catid=31:general&Itemid=46].
1.2 EPIDEMIOLOGY OF TUBERCULOSIS

According to the World Health Organization (WHO), there were an estimated 8.6 million incident cases of TB globally in 2012. Of these, 1.1 million were co-infected with human immunodeficiency virus (HIV) [WHO Report, 2013].

Kenya is on the list of 22 high-burden TB countries in the world and has the sixth highest burden in Africa and fifteenth in the world. In 2012, Kenya had approximately more than 89,000 new TB cases and approximately 9,500 retreatment cases [WHO Kenya Report, 2013].

1.3 PATHOGENESIS OF TUBERCULOSIS

The probability of developing active clinical TB after inhalation of an \textit{M. tuberculosis} aerosol from an infectious patient with active TB is very small, with an estimated lifetime risk of about 10% [Lawn and Zumla, 2011]. The clinical manifestations of TB represent a complex interaction between the causative organism, \textit{M. tuberculosis}, and the human host immune response [Schluger, 2005].

\textit{M. tuberculosis} usually enters the alveolar passages of exposed humans in an aerosol droplet, where its first contact is thought to be with resident macrophages. The bacteria are phagocytosed in a process that is initiated by bacterial contact with macrophage mannose and/or complement receptors. Surfactant protein A, a glycoprotein found on alveolar surfaces, can enhance the binding and uptake of \textit{M. tuberculosis} by upregulating mannose receptor activity. On the other hand, surfactant protein D inhibits phagocytosis of \textit{M. tuberculosis} by blocking mannosyl oligosaccharide residues on the bacterial cell surface, and it is proposed that this prevents \textit{M. tuberculosis} interaction with mannose receptors on the macrophage cell surface [Smith, 2003].

On entry into a host macrophage, \textit{M. tuberculosis} and other intracellular pathogens initially reside in an endocytic vacuole called the phagosome. If the normal phagosomal maturation cycle occurs, i.e., phagosome-lysosome fusion, these bacteria can encounter a hostile environment that includes acid pH, reactive oxygen intermediates, lysosomal enzymes, and toxic peptides [Smith, 2003]. The bacilli multiply in the macrophages, eventually killing them. Other blood-borne phagocytic cells, both macrophages and polymorphonuclear leucocytes, aggregate around the focus of infection and form a foreign body granuloma called the primary focus. Some bacilli are
transported to regional lymph nodes such as the mediastinal and paratracheal, where secondary lesions develop. A combination of the primary focus and the local lymphatic component is called the primary complex [Manson’s Tropical Medicine, 2009].

By 6-8 weeks after infection, the macrophages will have travelled to the draining lymph nodes and CD4+ T cells are activated. These T cells produce interferon-gamma (IFN-γ) which activates macrophages and enhances their ability to kill phagocytosed bacilli. However, *M. tuberculosis* is capable of surviving within macrophages because of components of its cell wall which inhibit the fusion of phagocytic vacuoles with lysosomes. Continuous T cell activation leads to the formation of granulomas which attempt to wall off the bacteria, and are often associated with caseous necrosis, which is caused by macrophage products such as lysosomes, enzymes and reactive oxygen species. Necrosis serves to eliminate infected macrophages and provides an anoxic environment in which the bacilli cannot divide. Thus the tissue injury may serve a protective function [Abbas *et al.* 2007].

Granuloma formation is a central event in the immune response against *M. tuberculosis*. While they restrict the spread of the infection, granulomas are space-occupying lesions that can damage surrounding normal tissues. The morphology of granulomas varies from person to person. In immunocompromised persons, there is poor granuloma formation, and there are therefore extensive areas of tissue necrosis containing large numbers of mycobacteria. In immunocompetent persons, indolent non-caseating granulomas containing few organisms are seen. In a majority of the cases, effective immune responses limit progression of the primary complex which heals by fibrosis and may eventually calcify. Alternatively, it may soften and enlarge with individual necrotic foci tending to coalesce, resulting in large areas of necrotic debris [Manson’s Tropical Medicine, 2009].
CHAPTER 2

2.1. DIAGNOSIS OF TUBERCULOSIS

The modern understanding of TB is usually traced to the work of Robert Koch. He developed staining techniques for *M. tuberculosis*, developed culture media in which to grow the organism, demonstrated the mode of transmission of the illness, and based on his understanding of the spread of the disease, recommended isolation of patients with TB [Schluger, 2005].

2.1.1. Tuberculin Skin Test (TST)

The Tuberculin skin test (TST), also known as the Mantoux test, is a clinical diagnostic tool. It measures a delayed-type hypersensitivity response to purified protein derivative (PPD), a crude mixture of antigens from the members of the *M. tuberculosis* (MTBC) complex and non-tuberculous mycobacteria (NTM). Unfortunately, the TST has low sensitivity in patients with either immune suppression or very advanced disease, and a low specificity in BCG-vaccinated individuals or in non-tuberculous mycobacteria (NTM)-exposed populations. Also, the administration and reading of the Tuberculin test requires a certain amount of expertise that, when lacking, may result in erroneous interpretations [Parsons et al, 2011].

2.1.2. Smear Microscopy

The earliest laboratory technique for diagnosis of TB was developed by Robert Koch in 1882 when he was able to stain bacilli in clinical specimen and observe them under a light microscope [Dorman S.E., 2012]. Bacilli can be stained using Ziehl-Neelsen (ZN) staining technique to demonstrate acid-fast bacilli (AFB). Although all mycobacterial species are acid fast, this assay is highly specific for *M. tuberculosis* in countries where TB is endemic because of the high burden of this disease [Parsons et al. 2011]. This technique is also simple, rapid and inexpensive. However, the technique is not sensitive, cannot distinguish *M. tuberculosis* from NTM, nor viable from non-viable organisms (TB Diagnostics and Laboratory Services).

Bacilli may also be detected by fluorescence microscopy. This involves staining the AFB with a fluorochrome such as auramine-O dye. Fluorescence staining is 10% more sensitive for the detection of pulmonary TB than conventional light microscopy. Also, fluorochrome-stained smears take less time to examine than those stained with the ZN method. Although FM can
reduce laboratory workloads, it has been difficult to implement it widely in low- and middle-income countries due to the high cost and complexity of the microscope and mercury vapour lamp lighting system, the need for a dark room, and perceived health risks associated with ultraviolet light exposure [Cuevas et al., 2011].

### 2.1.3. Culture of Tuberculosis

Culture of sputum specimens is used to distinguish between different mycobacterial species as well as to perform drug susceptibility testing and identification tests. However, *M. tuberculosis* divides every 15–20 hours, which is extremely slow compared with other bacteria (*Escherichia coli* divides every 20 minutes) [Lawn and Zumla, 2011]. It therefore requires egg-enriched media with glycerol and asparagines (Lowenstein-Jensen {LJ}) or agar-based media supplemented with bovine albumin (Middlebrook, 7H10 or 7H11). LJ is the most widely used medium for culture of *M. tuberculosis* [Revised National Tuberculosis Control Programme Training Manual, 2009].

There is also the semi-automated culture technique known as BACTEC 460. It uses a liquid broth such as Middlebrook 7H9, with carbon-labeled palmitic acid as a carbon source. The palmitic acid is metabolized by the organisms to carbon dioxide, and the rate of the increase of gas production is directly proportional to the growth of the organism [Parsons et al., 2011]. This test has the advantage of being faster than solid media but it is expensive and requires appropriate laboratory infrastructure such as nuclear waste disposal. This limits its use to reference laboratories only [Guillerm et al., 2006].

Another culture technique is the Mycobacteria Growth Indicator Tube (MGIT) system. It utilizes tubes containing the medium Middlebrook 7H9, growth supplements and antimicrobial agents which suppress the growth of contaminants. It also contains an oxygen-quenched fluorochrome embedded in silicone at the bottom of the tube. The free oxygen will be utilized during bacterial growth and the fluorochrome will fluoresce as it is no longer inhibited by the oxygen. Fluorescence can be read manually under an ultra-violet (UV) light or entered into an MGIT 960 instrument where it is incubated and monitored for fluorescence every sixty minutes [Parsons et al., 2011]. MGIT has the advantage of bulk processing than conventional culture, speed diagnosis in 7 days for sputum positive and DST in 8-12 days. However, the tubes are very expensive and the liquid media are known to have a high percentage of contaminations [Guillerm et al., 2006].
The microscopic-observed drug susceptibility (MODS) assay is another culture technique that is used for diagnosis of tuberculosis as well as testing for drug resistance. The test relies on three principles. First, that *M. tuberculosis* grows faster on liquid medium than on solid medium. Second, that characteristic cord formation can be visualized microscopically in liquid medium at an early stage. Third, the incorporation of drugs permits rapid and direct drug susceptibility testing concomitantly with the detection of bacterial growth. This test has been shown to outperform the gold-standard culture technique [Moore *et al.*, 2006].

### 2.1.4. Nucleic Acid Amplification Tests (NAATs)

The use of NAATs remains technically challenging. Despite being usually highly specific, NAATs have lower sensitivity. A positive NAAT is considered good evidence of infection but a negative result is not informative enough. Use of NAAT has not been recommended for sputum negative patients. While some NAA assays reported seem to work quite well there is very wide variability, even from resource-rich laboratories, making their use in the field uncertain [Guillerm *et al.*, 2006].

GeneXpert MTB/RIF assay is a self-enclosed, rapid PCR device [Wilson, M.L., 2011]. This test is favourable for use in field conditions as it reduces the amount of contact between the laboratory technician and the infectious sample. This is because the machine performs all functions of DNA amplification from specimen filtering, sonication to lyse the bacilli, released DNA collection and combination with the PCR reagents, amplification to target detection by fluorescence within two hours. This technique also tests for resistance of TB to rifampicin [Parsons *et al.*, 2011]. However, the assay requires uninterrupted and stable electrical power supply and yearly calibration of the cartridge modules [http://www.who.int/tb/dots/lab.pdf].

GenoType® MTBDRplus is a technique that is based on the DNA-STRIP technology. It permits the simultaneous molecular genetic identification of the *M. tuberculosis* complex, its resistance to rifampicin by detection of the most common mutations in the *rpoB* gene and its resistance to isoniazid by detection of the most common mutations in the *katG* and *inhA* gene from smear positive pulmonary clinical specimens or cultivated samples. The test is indicated for use in diagnosis of patients after treatment failure and relapse, patients with unknown anamnesis and originating from high prevalence areas of MDRTB, patients in high prevalence TB countries and
high-burden TB regions, patients from countries with high usage of the respective drugs and for screening purposes to develop country-specific tuberculosis action plans [http://www.hain-lifescience.de/en/products/microbiology/mycobacteria/genotype-mtbdrplus.html].

2.1.5. Immunodiagnostic Techniques

There are currently over forty rapid serologic TB tests. However, attempts to successfully develop tests that are sensitive and specific have not been successful. The tests that have been developed differ in a number of features including antigen composition, antigen source, chemical composition, extent and manner of purification of the antigen(s) and class of immunoglobulin (Ig) detected (IgG, IgM, or IgA). These tests are therefore not recommended for use as rapid diagnostic tests due to these features [Parsons et al, 2011].

Another immunodiagnostic technique is the interferon gamma (IFN-γ) release assays (IGRAs). These tests measure the IFN-γ release from T cells after stimulation by *M. tuberculosis*-specific antigens via an enzyme-linked immunosorbent assay (ELISA) or an enzyme-linked immunospot (ELISPOT) assay [Parsons et al, 2011]. The T-SPOT tuberculosis assay is a simplified ELISPOT technique. The test requires separation of peripheral blood mononuclear cells (PBMCs) from whole blood, and exposing them to the TB antigens for cytokines to be released by the PBMCs. This test is carried out in a microtitre plate and formation of an insoluble precipitate (spot) represents an individual cytokine-secreting T cell. Evaluating the number of spots obtained provides a measurement of the abundance of *M. tuberculosis* sensitive effector T cells in the peripheral blood [T SPOT, Oxford Immunotec].

Another IGRA assay is the QuantiFERON-TB Gold In-Tube, which is an ELISA. In this technique, whole blood is collected in QuantiFERON-TB Gold blood collection tubes, which include a Nil Control tube, TB Antigen tube, and a Mitogen Control Tube. The tubes are then incubated at 37°C for 16-24 hours. The tubes are centrifuged, plasma is removed, and the amount of IFN-γ measured by ELISA. A test is considered positive for an IFN-γ response to the TB Antigen tube that is significantly above the nil IFN-γ International units (IU)/ml value. The Mitogen-stimulated plasma serves as an IFN-γ positive control. A low response to Mitogen indicates an indeterminate result when a blood sample has a negative result to the tuberculosis
antigens [QuantiFERON-TB Gold. Cellestis]. These IGRAs are used to test for latent tuberculosis infections (LTBI).

2.1.6. Urine Lipoarabinomannan Test

Lipoarabinomannan (LAM) is a 17 500 dalton cell wall lipopolysaccharide specific for the genus *Mycobacterium*, released from metabolically active or degrading bacterial cells. Once in the bloodstream, LAM is filtered by the kidneys and can be detected in the urine intact. LAM-ELISA is a direct antigen sandwich immunoassay which detects LAM or LAM-containing bacteria. The test kit consisted of a 96-well ELISA plate pre-coated with LAM-specific antibody, blocked and sealed in a plastic pouch with desiccant. Urine samples are considered positive if the optical density (OD) at 450 nm is at least 0.1 above the signal of the negative control [Boehme *et al*, 2005].

2.1.7. Loop-Mediated Isothermal Amplification

Loop-mediated isothermal amplification (LAMP) is a simple, rapid and specific NAAT that is characterized by the use of six different primers specifically designed to recognize eight distinct regions on the target gene. The reaction process proceeds at a constant temperature using a strand displacement reaction. Amplification and detection of genes can be completed in a single step, by incubating the mixture of samples, primers, DNA polymerase with strand displacement activity and substrates at a constant temperature (about 65°C). It provides high amplification efficiency, with DNA being amplified 10⁹-10¹⁰ times in 15-60 minutes. Because of its high specificity, the presence of amplified product can indicate the presence of target gene [http://loopamp.eiken.co.jp/e/lamp/index.html]. Several other studies have been carried out to test whether LAMP can be used to detect TB. Different gene targets have been used, the most attractive being the *IS6110* gene as it gave higher sensitivity and specificity [Kohan *et al*, 2011].
2.2 JUSTIFICATION

The current gold standard diagnostic tool for TB is culture, which takes a minimum of 6 weeks to get a result. The LAMP assay promises to serve as a simple, rapid, specific and cost-effective point-of-care diagnostic tool. The assay is simple as it does not require highly trained laboratory technicians. It is rapid, as results are obtained in less than an hour. It is cost-effective as it does not require advanced laboratory technology. The technique can be adopted to achieve Goal 6 of the Millennium Development Goals, which is to combat HIV/AIDS, malaria and other diseases, including TB. The target of this goal is to halt and reverse the spread of tuberculosis by 2015 [Millennium Development Goals]. However, the progress has not been so good. One reason for this is that diagnosis for TB is challenging and inaccessible. LAMP has the potential to change this. According to the 2012 Progress Chart, Sub-Saharan Africa has been unable to achieve this target so far, as there are still high mortality rates. The report concluded that progress is insufficient to reach the target if the prevailing trends persist [Millennium Development Goals, 2012].

2.3 BROAD OBJECTIVE

To evaluate the performance of LAMP against culture on Lowenstein-Jensen for diagnosis of tuberculosis.

2.3.1 Specific Objectives

1. To evaluate the sensitivity of LAMP against culture on Lowenstein-Jensen for diagnosis of tuberculosis

2. To evaluate the specificity of LAMP against culture on Lowenstein-Jensen for diagnosis of tuberculosis

3. To determine the positive and negative predictive values of LAMP against culture on Lowenstein-Jensen for diagnosis of tuberculosis
CHAPTER 3

3.1. MATERIALS AND METHODS

3.1.1. Study Design

This study was a laboratory-based methods comparison study. LAMP assay was evaluated against culture on LJ for the diagnosis of TB. The assay involved amplification of the target gene, *IS6110*, and detection of the amplified gene. To diagnose TB, the test required six primers that recognized eight distinct regions of the *IS6110* gene. The assay was conducted at the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID). The culture test was performed at the National Tuberculosis Reference Laboratory (NTRL).

3.0.2. Sample Collection

The samples used were left over samples from the NTRL. The patients received their results from the Reference Laboratory. A total of 385 samples were collected. However, 25 cultures got contaminated. Thus, 360 samples were used for this study.

3.0.3. Sample size

The sample size was calculated using the following formula:

\[ N = Z^2 \times P (1-P)/C^2 \]

**Where:**

\( N \) = Sample size required

\( Z \) = z value of confidence level of 95% (1.96)

\( P \) = Prevalence of 50%, expressed as a decimal

\( C \) = Confidence interval of 5%, expressed as a decimal

Using the above formula, 384 samples were collected for the study.
3.0.4. Data Collection

After collection of samples, the results from the LJ culture, which was performed by the NTRL, were recorded onto a Microsoft Office Excel spreadsheet. The LAMP assay was conducted at UNITID and the results were also recorded on the spreadsheet.

3.0.5. Data Analysis

The results from the LAMP assay were compared to results from culture on LJ medium. A two by two (2x2) analysis table was created, as shown below, so as to demonstrate the true positives (TP), false positives (FP), true negatives (TN), false negatives (FN), sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Culture</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Total</td>
</tr>
<tr>
<td>LAMP</td>
<td>Positive</td>
<td>TP (a)</td>
<td>FP (b)</td>
<td>a+b</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>FN (c)</td>
<td>TN (d)</td>
<td>c+d</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>a+c</td>
<td>b+d</td>
<td></td>
</tr>
</tbody>
</table>

3.2. LABORATORY METHODS

3.2.1. Lowenstein-Jensen Medium Method

Before inoculation into the medium, the sample was homogenized and decontaminated using the Modified Petroff’s procedure. About 4 to 5ml of sputum was transferred to a centrifuge tube and double the volume of sterile 4% sodium hydroxide (NaOH) was added to the sputum aseptically. The caps of the tubes were tightened and the contents of the tube mixed well. Each tube was inverted to ensure that NaOH solution contacts all the sides and inner portions of the caps. The tubes were placed on a shaker and incubated at 37°C for 15 minutes.

The tubes were then removed from the incubator and 15ml of sterile distilled water was added. They were mixed well and centrifuged at 3000 g for 15 minutes. The tubes were then removed from the centrifuge carefully without shaking. The supernatant was discarded slowly into a container with 5% phenol solution. The sediment was washed with sterile distilled water and
centrifuged at 3000 g for 15 minutes, and the supernatant decanted. Using a wire loop, two slopes of LJ were inoculated from the sediment. The tubes were labeled and incubated at 37°C. Growth on the media was monitored for up to 8 weeks. Colonies of *M. tuberculosis* on LJ are rough, crumbly, waxy, non-pigmented and slow-growing [Revised Manual for *M. tuberculosis*. 2009].

3.2.2. LAMP Assay

Decontaminated sputum samples were collected from the NTRL. Mycobacterial DNA was extracted using QIAamp DNA mini kit using the protocol described in *artus® M. tuberculosis* RG PCR Kit Handbook.

The assay was used for diagnosis of TB by detection of the *IS6110* gene. There were a set of 6 primers, comprising 2 outer primers (F3 and B3), 2 inner primers (FIP and BIP), and 2 loop primers (FLP and BLP), as were described by Kohan *et al*. These primers recognized 8 distinct regions on the target sequence. Primer sequences used were as follows:

F3 - CCTAACCGGCTGTGGGTA  
B3 - CGAGTACGCCTTCTTGTTGG  
FIP (F1c + F2) - GACGTAGGCGTCGGTGACAAAGGCAGACCTCACCTATGTGTC  
BIP (B1c +B2) - GTCGCTTCCACGATGGCCACGGTCCAGATGGCTTGCTC  
FLP - TAGGCGAACCCTGCCCA  
BLP - TGGTCTCGACGCAGATC

The assay was conducted in a total volume of 25μl containing 0.2μM each of F3 and B3, 1.6 μM each of FIP and BIP, 0.8μM each of FLP and BLP, 20mM Tris-HCl (pH: 8.8), 10mM KCl, 10mM (NH₄)₂SO₄, 9mM MgSO₄, 1.4mM dNTP, 0.8M Betaine (Sigma-Aldrich), 8units of Bst DNA polymerase (New England Biolabs, USA) and 5μl DNA sample. The tubes were incubated at 65°C for 40 minutes in a heating block for amplification to take place [Kohan *et al*. 2011].

LAMP amplicons in the reaction tube were detected under ultra-violet (UV) light after adding 1.0 μl of 1/10-diluted original SYBR Green I (Invitrogen) to the tube and observing for fluorescence of the solution. The solution fluoresced green in the presence of LAMP amplicons, but remained orange with no amplification [Iwamoto *et al*. 2003].
3.3. ETHICAL CONSIDERATIONS

This study sought ethical approval from the Kenyatta National Hospital/University of Nairobi Ethics and Research Committee. The study also sought permission from the National Tuberculosis Reference Laboratory.
CHAPTER 4

4.1. RESULTS

A total of 360 samples were analyzed using LAMP and LJ culture. The results were then compiled on a Microsoft Excel spreadsheet. There were a total number of 360 samples that were analyzed. By LJ culture, 138 samples were positive and 222 samples were negative. LAMP tested 144 samples to be positive and 216 samples to be negative. The data was then compressed into a 2X2 table as is shown below:

Table 2

<table>
<thead>
<tr>
<th></th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAMP</td>
<td>136</td>
<td>8</td>
<td>144</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>214</td>
<td>216</td>
</tr>
<tr>
<td>Total</td>
<td>138</td>
<td>222</td>
<td>360</td>
</tr>
</tbody>
</table>

Sensitivity, specificity and predictive values were then calculated as follows:

Sensitivity = \[ \frac{TP}{TP + FN} \times 100 = \frac{136}{136 + 2} \times 100 = 98.6\% \]

Specificity = \[ \frac{TN}{TN + FP} \times 100 = \frac{214}{214 + 8} \times 100 = 96.4\% \]

PPV = \[ \frac{TP}{TP + FP} \times 100 = \frac{136}{136 + 8} \times 100 = 94.4\% \]

NPV = \[ \frac{TN}{TN + FN} \times 100 = \frac{214}{214 + 2} \times 100 = 99\% \]

Sensitivity = 98.6%

Specificity = 96.4%
PPV = 94.4%

NPV = 99%

The study showed a sensitivity of 98.6%. This means that 98.6% of the study population was declared to be diseased and 1.4% of the study population was falsely declared to be disease-free by the test. The study showed a specificity of 96.4%, which means that 96.4% of the study population was declared to be disease-free, while 3.6% was falsely declared to be diseased by LAMP. The study also showed a PPV of 94.4% and an NPV of 99%. Thus when LAMP has a positive result, we are 94.4% certain that the patient has TB and if a negative result is received, we are 99% certain that the patient does not have TB.

SYBR Green I was added to the mixture and the tubes placed under UV light. A green fluorescence indicated a positive result while no fluorescence indicated a negative fluorescence [Fig. 1].

Sensitivity is the ability of a clinical test to correctly identify those patients with the disease while specificity is the ability of a clinical test to correctly identify those who do not have the disease (Lalkhen and Mccluskey. 2008). This study shows a sensitivity of 98.5% in culture – positive samples and a specificity of 96.3% in culture-negative samples.
Figure 1
CHAPTER 5

5.1 DISCUSSION

One of the important reasons for failure to control TB is the lack of affordable simple diagnostic methods that have better sensitivity and specificity than conventional methods commonly used in clinical mycobacteriology laboratories [Kohan et al., 2011]. PCR and other NAAT methods for TB have great advantages of speed (compared to culture) and sensitivity (compared to microscopy). Furthermore, recently developed real-time assays may generate results in less than an hour, a key requirement for point-of-care testing. The utility of current NAAT methods is limited, however, by their cost and complexity, particularly in countries where TB is endemic. LAMP has inherent properties that make amplification and detection possible in one uninterrupted process, with no need to open the amplification vessel or any need for a luminometer or other detection device. The assay utilizes a single polymerase that is active at relatively high isothermal amplification temperatures, diminishing the likelihood of nonspecific priming. The use of six primers increases specificity at the same time that it enhances speed [Boehme et al., 2005].

LAMP has several advantages in comparison with PCR. LAMP amplifies DNA under isothermal conditions, requiring only a regular water bath or heating block for maintaining the temperature at 66°C, and make it more economical and practical than PCR. In addition, LAMP is more effective and rapid than conventional PCR. In PCR, nearly 3 h is required for the detection and post PCR analysis, while LAMP assay require less than 1 h. LAMP produces extremely large amounts of amplified products and enables simple detection methods such as visual judgment by the turbidity or fluorescence of the reaction mixture, which is kept in the reaction tube. The visual detection eliminates the need for time-consuming electrophoresis and costly specialized equipment [Kohan et al., 2011].

In this study, a total of 360 sputum samples were studied by culture on LJ and LAMP. Culture on LJ was performed by NTRL and took a maximum of eight weeks to get a result. LAMP was conducted at UNITID. LAMP reaction begins with the mixing of buffer, primers, DNA lysates, and Bst polymerase in a tube. The target gene that was amplified was the IS6110 gene. The mixture was then incubated at 65°C for 40 minutes. The only equipment needed for the LAMP
reaction is a regular laboratory water bath or a heat block that furnishes a constant temperature of 65°C.

A multicentre study on *M. tuberculosis* detection showed the feasibility of using the LAMP method in developing countries (Boehme *et al*, 2007). The study evaluated a prototype LAMP assay targeting the *gyrB* gene and using a simplified manual DNA extraction method. The sensitivity of LAMP assay targeting the *gyrB* gene was 88.2%. The sensitivity of this study is 98.5%. Another study by Pandey *et al* (2008) showed the feasibility of LAMP assay targeting the *rrs* gene; the sensitivity of LAMP was 100% in culture positive sputum samples and specificity was 94.2% in culture negative sputum samples. In this study, the specificity of LAMP was 96.3%. Thus this study shows high specificity and sensitivity in comparison with LAMP methods used in previous studies.

LAMP has demonstrated to be a point-of-care tool as it can provide a result in less than an hour. The current point-of-care tool used is smear microscopy. However, it has low sensitivity. Culture has high sensitivity and specificity but takes a longer period of time to get a result and is not available as a point-of-care tool as it requires specialized equipment. LAMP has high specificity and sensitivity, and gives results within a short period of time.
5.2 CONCLUSION

This LAMP assay is an NAA method that allows direct identification of *M. tuberculosis* in processed sputum samples. Due to its ease of use in the laboratory, use of inexpensive equipment and its high sensitivity and specificity, this assay may be adopted in Kenya as a point-of-care diagnostic tool to the identification of *M. tuberculosis* without the use of culture.
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