UNIVERSITY OF NAIROBI

DEVELOPMENT AND VALIDATION OF RAPID MOLECULAR ASSAYS FOR DIAGNOSIS AND SURVEILLANCE OF *WUCHERERIA BANCROFTI* IN HUMAN BLOOD AND MOSQUITO VECTORS IN KENYA

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I80/50034/2015

A thesis submitted in fulfillment of the requirements for the award of degree of Doctor of Philosophy in Applied Parasitology of the University of Nairobi.

November, 2022

DECLARATION

I, do hereby declare that this thesis is my original work and has not been presented for a degree or any other award in any other university.

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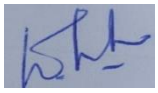
1. **Dr. Wilkinson Mutahi** **Signature:**  **Date :** 30/11/2022
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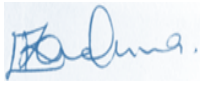
2. **Dr. Esther Kanduma** **Signature:**  **Date:** 30/11//2022
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DEDICTATION

First and formost, I dedicate this work to the Almighty God who has seen me through thick and thin moments. Having taken care of me, providing for me, securing me when in the field in the most insecure County-Tana River, receive all the glory and honor. Much dedication to my loving and caring husband Dr. Patrick K. Malonza for his support and encouragement throughout the study and for always being there for me. Dear, you have endured my absence while out in the field and in the laboratory, much blessings. My children; son Eric Muendo, daughters Irene Kavua and Annika Martha for the encouragements and patience when I was busy not able to attend to your needs. To my parents, late dad Kimanzi Isika who left me while I was still pursuing my PhD studies, my mother, Martha Kimanzi for praying for me and being patient with me when situations were tough and unable to attend to you or even visit. My siblings; brothers and sisters for standing by my side. Not forgetting my spiritual father Pastor Joshua Wambua, when I felt like giving up on the way, your encouragements and prayers energized me to move on. Reverend, Dr. Mutahi, you made a dedication prayer for me in your office which assured me that I was going to complete my studies. The journey was not easy but through it all, I made it by every bit of support you all offered. Much blessings to you all.

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LIST OF ACRONYMS AND ABBREVIATIONS

LAMP	Loop-mediated isothermal amplification
SDA	Strand displacement amplification
HDA	Helicase-dependent amplification
PCR	Polymerase chain reaction
NASBA	Nucleic acid sequence-based amplification
IMDA	Isothermal multiple displacement amplification
SMART	Signal mediated amplification of RNA technology
SPIA	Single primer isothermal amplification
RCA	Rolling circle amplification
TMA	Transcription mediated amplification
cHDA	Circular helicase-dependent amplification
DNA	Deoxyribonucleic Acid
WHA	World health assembly
EDTA	Ethylenediaminetetra acetic acid
ANOVA	Analysis of variance
FTS	Filariasis Test Strip
MDA	Mass drug administration
WHO	World health organization
OC	Oligochromatographic
GPELF	Global program for elimination of lymphatic filariasis

RPA	Recombinase Polymerase Amplification
MMDP	Morbidity Management and Disability Prevention
KEMRI	Kenya Medical Research Institute
CBRD	Centre for Biotechnology Research and Development
POC	Point-of-Care

ABSTRACT

Background: Lymphatic filariasis is caused by *Wuchereria* and *Brugia* species. Elimination control programme aims at eliminating the disease as a public health problem. Point-of-care, affordable, user friendly and accurate diagnostic tools are needed. Molecular characterization of *Wuchereria bancrofti* 18S rRNA gene was characterized for diagnostics targets analysis. Loop-mediated and Helicase dependent isothermal amplification assays were developed, evaluated and validated against PCR assay.

Materials and methods: Human blood samples and mosquito were collected from Malindi, Tana River and Busia Counties which have a high prevalence of lymphatic filariasis. Whole genomic DNA were extracted and *W. bancrofti* 18S rRNA fragments were amplified by PCR and sequenced. LAMP and HDA assays were used for screening and detection of *W. bancrofti* DNA in human blood and mosquitoes. Kappa statistics were calculated to determine congruence between the two tests. The sensitivity and specificity of LAMP and HDA assays were compared using 2X2 contingency table. Chromas version 2.6.5 and BioEdit softwares were used for manual editing and multiple sequence alignment of the 18S rRNA nucleotide sequences. Phylogenetic analysis was done in MEGA 7.

Results: Malindi and Tana River 18S rRNA isolates had a mean genetic distance of 0.99. Percent identity of the 18S rRNA nucleotide sequence with GenBank reference sequences ranged from 79-98%. The sensitivity of LAMP assay was 92.3%, while that of HDA was 76.6%. The specificity of the LAMP assay was 97.3% and that of HDA was 93.7%. Kappa statistics was at 0.84 and 0.67 for LAMP and HDA respectively.

Conclusion: *Wuchereria bancrofti* 18S rRNA gene were highly conserved among the 14 isolates from Malindi and Tana River and thus, suitable for diagnostic targets. LAMP assay was found to be more sensitive and comparable to PCR than HDA.

Recommendations: More genetic studies to be undertaken to establish the degree of diversity in *W. bancrofti* strains in Kenyan endemic regions. LAMP assay should be adopted in local health clinics at endemic regions for diagnosis of lymphatic filariasis.

Key words; genetic, characterization, variation, LAMP, HDA, *Wuchereria*, *bancrofti*, isotherma

CHAPTER ONE: INTRODUCTION

1.1: Public health significance of lymphatic filariasis infections

Lymphatic filariasis is an infectious vector borne disease of filaridae family. The helminths belonging to genera *Wuchereria* and *Brugia* parasites are transmitted by mosquito species (WHO, 2015). The disease affects over 68 million people worldwide, of which 90% of the infections are due to the parasites of *W. bancrofti*. The disease causes lymphedema, elephantiasis and genital deformities that impair the lymphatics resulting to long-lasting disability (WHO, 2017; Hemalatha *et al.,* 2016).

Mosquito genera of *Anopheles*, *Aedes*, *Culex* and *Mansonia* species transmit filarial parasites (WHO, 2013). Human beings get infected when mosquitoes deposits infective larvae stage three (L_3) under the skin during feeding (Simonsen, 2009). Occassionally the victims suffer from periodic attacks of high fever due to bacterial infections in the affected part. These symptoms lead to considerable incapacitation to the affected individuals leading to social and psychological stress as well as loss of income due to work days lost (Zeldenryk *et al*., 2011).

For diagnosis, microscopy has been the gold standard method for detection and identification of microfilariae in human blood and in mosquito vectors (Mcmahon *et al.,* 1979). Manual dissection and examination of *microfilariae* in mosquitoes has been used in determination of infection and infectivity rates in the vectors (Cartel *et al.,* 1992). Circulating filarial antigens (CFA) and IgG4 antibody detection markers are also used (Weil and Pani, 2000). *Wuchereria bancrofti* DNA in the host and in vectors is detected by polymerase chain reaction (PCR) molecular assays (Farid *et al*., 2001). These advances in diagnosis are timely in view of the diagnosis need for the reduction of economic and social burdens of filariasis.

In Kenya, *W. bancrofti* is endemic in Coast region along the Indian Ocean where 3.4 million people are estimated to be at-risk of infection with 1 million infections (Moraga *et al.*, 2015).

1.2: Social-economic burden

Morbidity due to chronic stages of filariasis severely affects the social and economic growth in endemic areas (Martindale *et al.*, 2014; Zagaria and Saviola, 2002). Lymphatic filariasis contribute to 5.25 million disability- adjusted life years (DALYs) in 2000 before the start of MDA programs. In 2016 this had dropped to 2 million DALYs after several round of treatment (WHO, 2016). The economic burden due to filariasis in the US amounted to $ 5.765 billion annually (Christopher *et al*., 2020) with a loss of almost US $1 billion in Africa annualy (Haddix *et al*., 1999). The disease causes huge burden to individuals, households and to the government-funded and private healthcare systems (Ramaiah *et al*., 1996: 2000). Repeated filarial attacks known as adenolymphangitis (ADL) are common with the victims which hinder them from enganging on their day-to-day activities (Kwaeteng *et a.l*, 2019; Dai *et al.*, 2016; Mcpherson *et al.*, 2006). The largest portion of economic burden (US $114.69) is associated with chronic manifestations and acute attacks (Christopher *et al.*, 2020; Krol *et al 2013*; Chu *et al.*, 2010) with hydrocele cases causing 83% of economic loss (Lu *et al.*, 1988). Youths are the most affected by hydrocele leading to the huge economic loss as they contribute more to economic development. The medical management of hydrocele are a burden to health systems and to the family and care givers (WHO, 2018). People with *W. bancrofti* suffer from pain, disfigurement and sexual disability although the disease is not considered deadly (Ahorlu *et al.*, 1999). Both men and women victims become physically challenged and social stigmatization is high. The disease is associated with shame and taboos which affect marriages or becomes impossible for the youth to marry (Gyapong *et al.*, 1996b).

1.3: Lymphatic filariasis control

World Health Assembly Resolution 50.29 of 1997 resolved to eliminate lymphatic filariasis by the year 2020 (WHO, 2018; 2016). This is based on 2 pillars (i) To have the entire at-risk population treated for transmission interruption in endemic communities (ii) To initiate morbidity management and disability prevention programmes. Hydrocoele cases were estimates at 25 million and 15 million people with lymphedema on a baseline survey of filariasis. By the year 2020, atleast 36 million persons still have chronic disease manifestations (WHO, 2020). Though a significant progress has been realized by the GPELF through the yearly cycles of mass drug administration (MDA), there are areas of hotspots that persit after 10 or more rounds of treatment in some endemic countries (Biritwum *et al*., 2016). By 2018, MDA programmes were not needed in twenty-four (24) of the initial 83 lymphatic filariasis endemic countries which were carrying out post treatment surveillances. There was a drop from 1.4 billion in 2011 to 893 million in 2018 people who required MDA (WHO, 2016; 2018; 2021). Pragrammes need to integrate MMDP activities to attain the second goal of elimination. Minimum recommended package services to manage chronic cases should be incorporated in health care systems in endemic regions.

National programme for elimination of lymphatic filariasis in Kenya launched in 2002 in Kilifi, Malindi and Kwale regions. By 2011, only four rounds of MDA had been given in some districts while other districts like Tana River had received only one round (Njenga *et al*., 2011). Though treatment had not been annually consistent, there had been a significant decline in transmission (Njenga *et al*., 2017). A continuous comprehensive assement of control programmes is necessary to assess the impact of the programmes and determine how to up-scaled the activities to achieve the elimination goal. The review of the programmes will give guidelines on the need for (1) re-mapping and (2) in identifying a

suitable tool for detection of continued transmission during post-MDA surveillance or validation phase (Srividya *et al*., 2019). Currently, efficient and accurate diagnostic tools capable of point of care detection of *W. bancrofti* in a field set up and at a reduced cost are lacking.

With the need for improved *W. bancrofti* diagnostic tools in mind, this study was conceived to genetically characterize *W. bancrofti* 18S rRNA gene for diagnostic targets and develop and optimize LAMP and HDA methods as point -of - care tools for diagnosis of *W. bancrofti* DNA in humans and vectors. Florescence dyes were evaluated for amplicon detection.

1.4: Problem statement

Wuchereria bancrofti transmission in host and vectors have reduced following intensive control interventions. This has led to low infection levels that are not easily detectable with the available diagnostic methods. The parasite genetic changes as a result of multations may lead to changes in diagnostic targets. For instance, microflariae detection by microscopy requires blood collection at night between 2200 and 0200 hours (Singh & Urhekar, 2013), this practice is not readily acceptable in some communities as they associate with evil worship or beliefs (Lucena *et al*., 1998; Ramzy, 2002). Some endemic areas in Kenya are prone to insecurity and not suitable for working at night. In addition, microscopy is time consuming, requires qualified personnel and the procedure is tedious in case of epidemiological surveys. The rapid antigen test by Immunochromatographic card test- ICT kits have been reported to have lost sensitivity in low microfilaraemic areas, the kits are also unavailable and this has limited their use in post elimination surveillance (WHO, 2011; Schuetz *et al.,* 2000). Filarial Test Strip (FTS) have replaced ICT kits though they are not locally available and the cost of importation is high. Molecular assays including PCR are sensitive and accurate but they have not been widely used in a field set

up because they require sophisticated laboratories associated with expensive equipments, reliable power supply and well qualified technical staff. These factors pose a challenge for the development of appropriate survey methods to help in assessing transmission interruption

1.5: Study justification

Though lymphatic filariasis had been targeted by global and national programmes for elimination in endemic countries by the year 2020, elimination goal was not achieved by most countries as it was anticipated. The new WHO-Neglected Tropical Diseases roadmap 2021-2030 (WHO, 2021) targets filariasis for elimination from endemic regions by the year 2030.

Kenya being one of the endemic countries started MDA programmes in 2002 and since then, there has been a good progress in the control interventions. From the year 2017, there has been consistent annual treatments that have significantlly reduced the transmission index, thus, there is need for accurate mass diagnosis of *W. bancrofti* infections to assess the success of the control programmes. This require diagnostic tools which are accurate, reliable, easy to run, affordable and that require less equipment.

This study therefore, sought to genetically characterize and phylogenetically analyse *W. bancrofti* 18S rRNA gene for diagnostics targets and to assess the use of LAMP and HDA isothermal molecular methods as point-of-care diagnostic tools for *W. bancrofti* parasites in both mosquitoes and human population.

In this study, Malindi and Tana River study sites were selected because they had a high initial infection prevalences. Busia is a presumed endemic region for *W. bancrofti* infections and hence included for the Assays validations.

1.6: Hypothesis

1.6.1: Null hypotheses

1. The 18S rRNA gene is not appropriate target for *Wuchereria bancrofti* parasite detection.
2. Loop mediated and Helicase dependent isothermal amplification assays are not superior to PCR and FTS methods in diagnosis of *W. bancrofti* parasites.
3. The accuracy and point- of- care validations of Loop mediated and Helicase dependent isothermal amplification assays coupled with a rapid amplicon detection is not superior to that of PCR and gel electrophoresis methods for the diagnosis of *W. bancrofti* parasites.

1.7:1: General objective

1. To characterize and phylogenetically analyse *W. bancrofti* 18S rRNA gene, design and to evaluate the accuracy of LAMP and HDA isothermal amplification methods for detection of *W. bancrofti* in blood samples and mosquito vectors in Tana River Delta, Malindi and Busia, Kenya.

1.7.2: Specific objectives

1. To characterize and phylogenetically analyse *W. bancrofti* 18S rRNA gene for molecular assays development.
2. To design and optimize LAMP and HDA assays in amplification of *W. bancrofti* DNA in human blood and mosquitoes.
3. To validation the most appropriate amplification method between LAMP and HDA assays using human blood and mosquitoes in a field set up

CHAPTER TWO: REVIEW OF THE LITERATURE

2.1: Background Information

2.1.1: Lymphatic filariasis epidemiology

Filariasis is a dire plague which invokes morbidity, disability and psychosocial stigma. Though it is not a deadly disease, it is the second cause of long-term disability after eye blindness (WHO, 2017). Fenwick (2012) had estimated filariasis to cause 5.17 disability adjusted life years (DALY's). It is one of the most neglected diseases in the tropics (NTD) and affects poor of the poorest communities. The disease is of a major medical and social importance and in its chronic forms it led to disabling conditions such as elephantiasis and genital deformities (WHO, 2020). Species of *W. bancrofti, B. malayi* and *B. timori* are known to cause the disease (WHO, 2016). The disease is endemic in many regions in 83 countries including parts of Southeast Asia, Sub-sahara Africa, Islands of Pacific and Latin America. A total of 120 million people worldwide suffer from filariasis with 1.2 billion people at risk (Chandy *et al*., 2011; WHO, 2016; Small *et al*., 2013). Thirty-six (36) million people have microfilaria, 25 million suffer from hydrocele, and 15 million from debilitating lymphoedema (Adhikari *et al*., 2015; Chandy *et al*., 2011).

2.1.2: Global situation and elimination progress

A total of over 7.1 billion treatments have been used by the control programmes since 2000. This has been possible through the co-ordinated efforts of various partners including ministries, donors, research communities and global programme to eliminate lymphatic filariasis (WHO, 2017). Published guidelines are available on stopping MDA and elimination verifications. China and Korea were the first countries to eliminate filariasis in 2007 (De-jian *et al*., 2013; Cheun *et al*., 2009). London declaration, 2012 was adopted globally and this led to reinforced commitment to elimination. By October 2018, sixteen

(16) countries and territories were formally declared by WHO to have attained the elimination goal (WHO, 2019).

Ten more countries are under surveillance for elimination certification. Despite the intensified control measures, 49 countries still require chemotherapy of which 15 of these countries had not started MDA by the year 2018 (WHO, 2021: 2019; Kamgno and Djeunga, 2020). Despite these shortcomings, the disease prevalence declined significantly from 199 million individuals in the year 2000 to 51 million individuals by the year 2017 (Kamgno and Djeunga, 2020). Even with the global prevalence reduction, there are focal areas in Africa and Southeast Asia that still have not attained the required threshold for elimination. Most African countries still have a high prevalence due to initial prevalence and the fact that most of them started control programme recently. The above limitations warrant more mapping and implementation coverage to accelerate progress towards elimination (Kamgno and Djeunga, 2020).

In Kenya, lymphatic filariasis is endemic in coastal region, elimination programmes were launched in 2002 in Kilifi County as the first intervention unit with mass drug administration of 6 mg/kg of Diethylcarbamazine and 400mg of Albendazole. The treatments were extended to include Malindi and Kwale regions which received MDA in 2003, 2005 and 2008. Annual MDA were not sustainable because of financial and administrative challenges. Tana River and Lamu Counties were included for the programme in 2011 while Taita Taveta received its initial round in 2015. In 2015, Njenga and colleaques, (2017) carried out surveys in sentinel sites of Lamu, Tana River, Kilifi, Kwale and Taita Taveta. An overall prevalences of circulating filarial antigen (CFA) of 1.3% in Kilifi and < 1.7% in Kwale County were found. Therefore, there is need for additional rounds of MDA in these counties (Njomo *et al.*, 2020; Njenga *et al.*, 2017).

Mombasa County was included in the control programmes in 2016 with Kenya achieving 100% geographical coverage.

The 2018 predications in Africa highlighted sub national variations in infection prevalences that persisted in West and Central Africa. Even with these predictions, data to support transmission potential in areas initially considered non-endemic such as northern Kenya is lacking (Kamgno & Djeunga, 2020). Other recent reports showed that Western Kenya, specifically Busia County is endemic for filariasis (Kinyatta and colleaques un-published data, 2020).

Figure 2.1 shows areas of filariasis endemicity worldwide and control program activities since the launch of the GPELF in 2000.

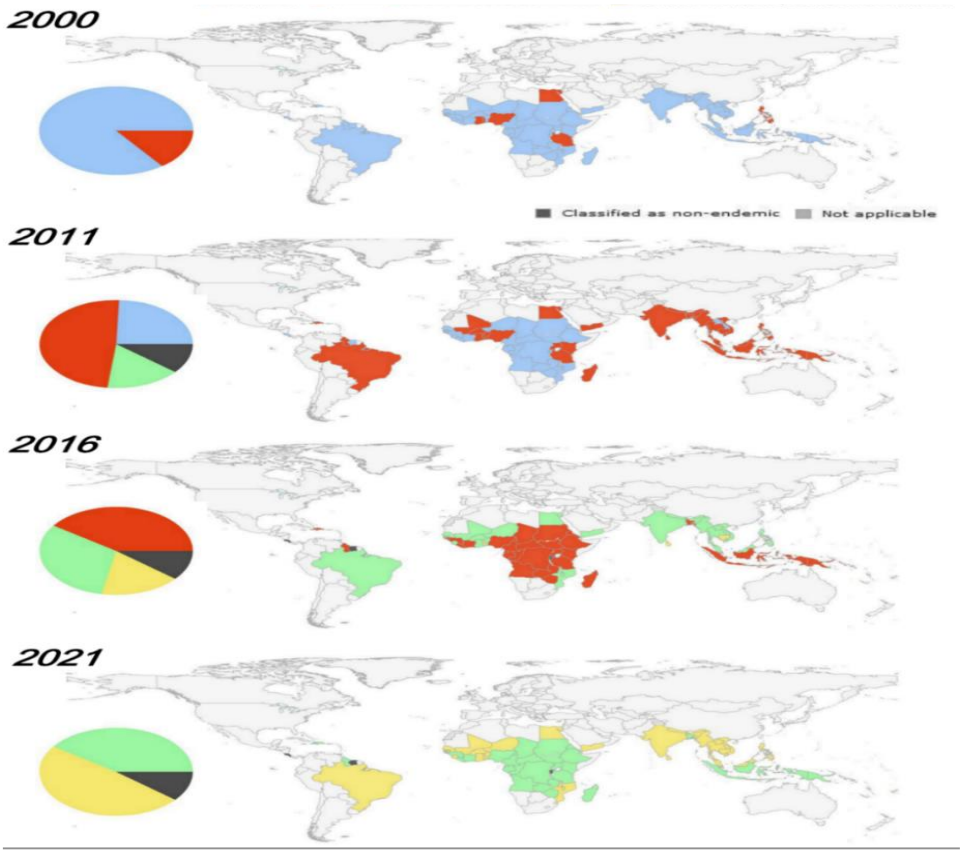


Figure 2.1: World Map showing world–wide Mass Drug Administration programme activities towards control of lymphatic filariasis progress between 2000-2021.
Programme control activities are described in different colours as follows**:**

Shows regions carring out mapping, regions carrying out MDA, regions carrying out post MDA surveillance; regions verified for transmission interruptions; regions classified as non endemic. **Source:** *https:// doi. org/ 10.1371/ journal. pntd. 0003328.g005.*

2.1.2.1: Why elimination of filariasis was not achieved in the year 2020

World health organization recommend a strategy of annual MDA of single or combined dose for 5 to 6 years, this is to interrupt the transmissions of lymphatic filariasis within a population. However, studies have found that period of treatment was not enough to interrupt transmission in all the countries tested (Wilson *et al.*, 2016; Endeshaw *et al*., 2015). This proves that, there is a need for more rounds of MDA and vector control measures. Improved MDA coverage and adequate monitoring is necessary for

implementation success (Njomo *et al*., 2020). Persistence hotspots and continued transmission even after more than 12 rounds of MDA were found to significantly be linked to high baseline antigen or microfilariae prevalence and non-compliance to MDA (Biritwum *et al.,* 2017; Shamsuzzaman *et al.,* 2017). This implicates that the number of MDA rounds required to reach threshold of <1% microfilariaemia prevalence or 2% antigen prevalence cannot be used for all endemic areas. Due to the highlighted reasons, WHO 2018 guidelines recommend 3-drug regimen comprising of Ivermectin, Diethylcarbamazine, and albendazole (IDA) to achieve elimination. (WHO, 2018; 2017). It was reported that triple drug (IDA) dose taken once rapidly cleared all microfilariae blood and the patients remaine amicrofilaraemic for at-least 2 years. This suggested that sterilization or death of adult worms had occurred (Thomsen *et al*., 2016). Studies by King *et al*. (2020) and Irvine *et al.* (2017*)* demonistrated that, a single triple drug therapy dose given to 65-90% coverage of people in the community was sufficient to interrupt transmission and therefore eliminate the disease. Therefore, due to this drug dose expansion, many eligible countries will not require treatment by the year 2030. However, there are challenges in getting the appropriate MDA monitoring and tool for the three-drug regime.

2.1.2.2: Morbidity management and disability prevention

This strategy is the second in the twin components of the GPELF (Das, 2002; WHO, 2011) that recommends morbidity and disability management essential services in health care services. 34 endemic countries have implemented MMDP (WHO, 2017) and thus it has lagged behind. Jullien *et al*. (2011) showed that morbidity due to filariasis can be reduce within 4-5 months if MMDP measures are in place. Surgical interventions can also alleviate hydrocoele cases and the life benefits of hydrocelectomy exceeds the cost of repairing hydrocoeles with the victims getting a relief for 2-4 years (Betts *et al*., 2020; Sawera *et al*.,

2020). Skin hygiene, excercises and elevation of affected limbs can reduce and prevent progression of the disease and acute inflammatory episodes. If there were proper integration of MMDP and MDA, majority of endemic countries would have elimination filariasis by the year 2020.

2.1.3: Etiology of *W. bancrofti*

Elephantiasis is caused by thread like-filarial nematode of the family Filaridae. *Wuchereria bancrofti* is the most common in Asia and Africa causing 90% of infections all over the world (WHO, 2019). The worms get in to the lymphatic system and form nests imparing normal flow of lymph fluid. Fertilized female worms produce a huge number of microfilariae which circulate in the blood streams of the host, the female life span is 6-8 years. (WHO, 2013). Mosquitoes ingest microfilariae when taking blood meal from the host. In the mosquito, the microfilariae undergo some developmental changes, whereby L_1 molts to L_2 and L_2 molts to L_3 which is the infective stage. Microfilariae (L_3) migrate to the vector's thorax and eventually into the mouth part ready for injected on the skin and get to body's circulation system and eventually to the lymphatic vessels where they mature to adults (Wilke & Marrelli, 2015). When death of the worms occurs, they calcify in the lymphatic system causing

blockage and swellings. Figure 2.2 shows microfilariae developmental stages in mosquitoes and the host.

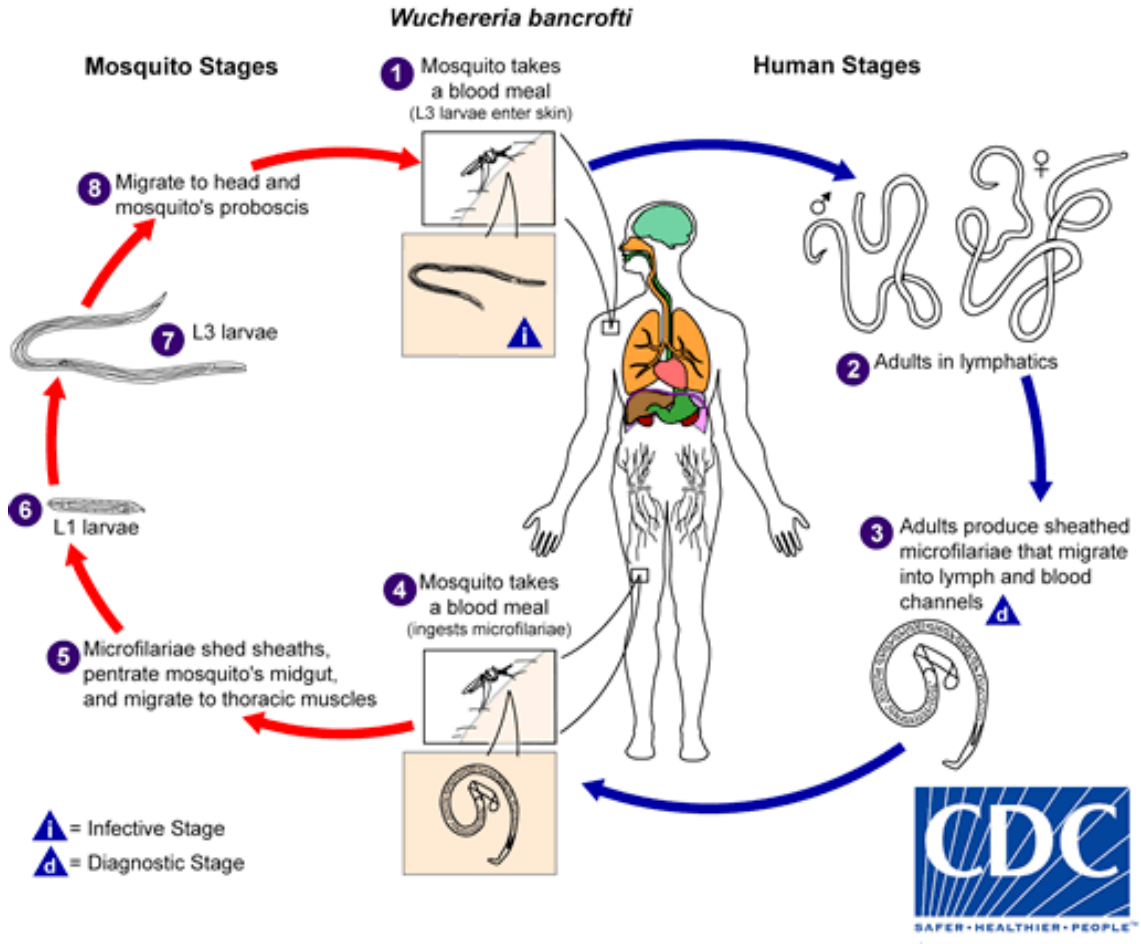


Figure 2.2: Illustration of *W. bancrofti* life cycle
Source: *http://ww.dpd.cdc.gov/dpdx.2010*

2.1.4: Transmission of lymphatic filariasis: Mosquito vectors

Culex, Anopheles, Aedes and *Mansonia* species are main vectors of filariasis (Kinyatta *et al*., 2018; Moraga *et al*., 2015). They are also important vectors of *P. falciphurum*, yellow fever and chikungunya (Stoops, 2010). Nocturnal *W. bancrofti* in coastal Kenya is transmitted by *Culex quinquefasciatus* (Kinyatta *et al*., 2018; 2011). In rural Africa, *Anopheles gambiae* and *An. funestus* play significant role (Kasili *et al*., 2009). Species of *Aedes* genera are major vectors for diurnal sub-periodic *W. bancrofti* and *B. timori*, while *B. malayi* is transmitted by *Mansonia species*.

2.2: Characteristics of vectors of lymphatic filariasis

2.2.1. *Culex* genus

Culex species have no postspiracular setae a feature that distinquishes it from the other genera. They have short maxillary as compared to the proboscis which is at an angle to the body part. The species is known for breeding and feeding in foulest water especially water canals and pit latrines, thus associated with urban area transmissions (Meyrowitsch *et al.*, 2011; Kinyatta *et al*., 2018). *Culex* mosquitoes feed at dusk and dawn having different preferences with respect to their hosts. Defferential morphological features of *Culex quinguefaciatus* mosquito are shown the figure below.

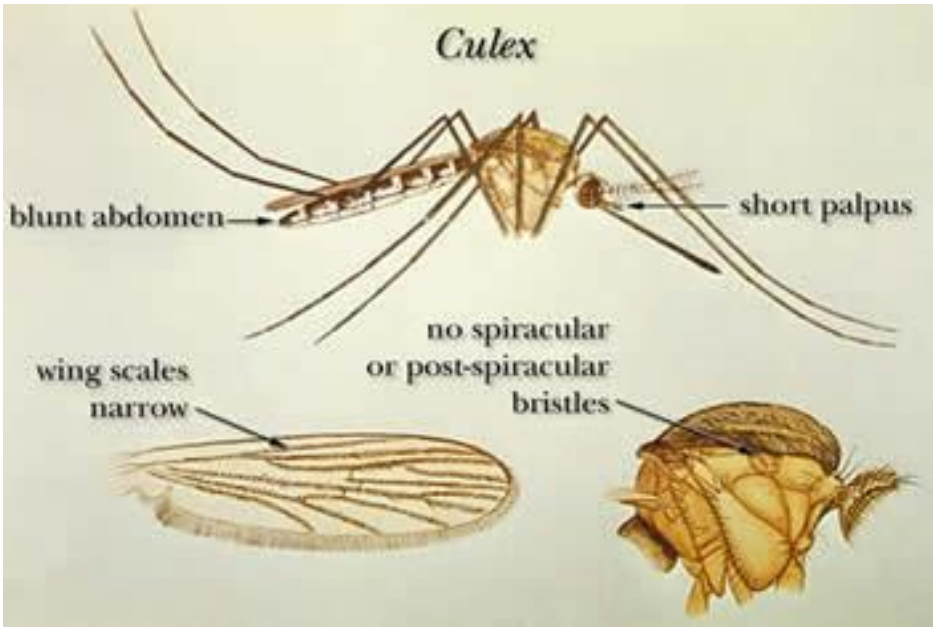


Figure 2. 3: Image of *Culex quinguefaciatus* mosquito

The head and the wings have important morphological features for identification**.** The wings scales are narrow. There is no spiracular or post-spiracular bristles. This species has blunt abdomen. **Source**: *https://www.cdc.gov/mosquitoes/gallery/index.html*

2.2.2: *Anopheles* genus

The palps and proboscis of Anopheles mosquitoes are both long and these are features commonly used for identification. The mosquitoes in this genus have dark body coloration with black and white scales on the wings. The mosquito rest at 90ºC position. They also have a specific selection site for oviposition (Lourena & Ferrell, 2019). The mosquitoes in this genus exist as Zoophilic and afew are anthropophic and are either endophagic, preferring to take their blood meal indoors. Adding further heterogeneity to this genus, some anopheline species are endophilic while others are exophilic. Anopheles species prefer clean water for ovipostion unlike *Culex* and *Aedes* species (Southgate & Bryan, 1992). The Figure 2.4 shows an adult Anopheles mosquito with distigushing morphological features.

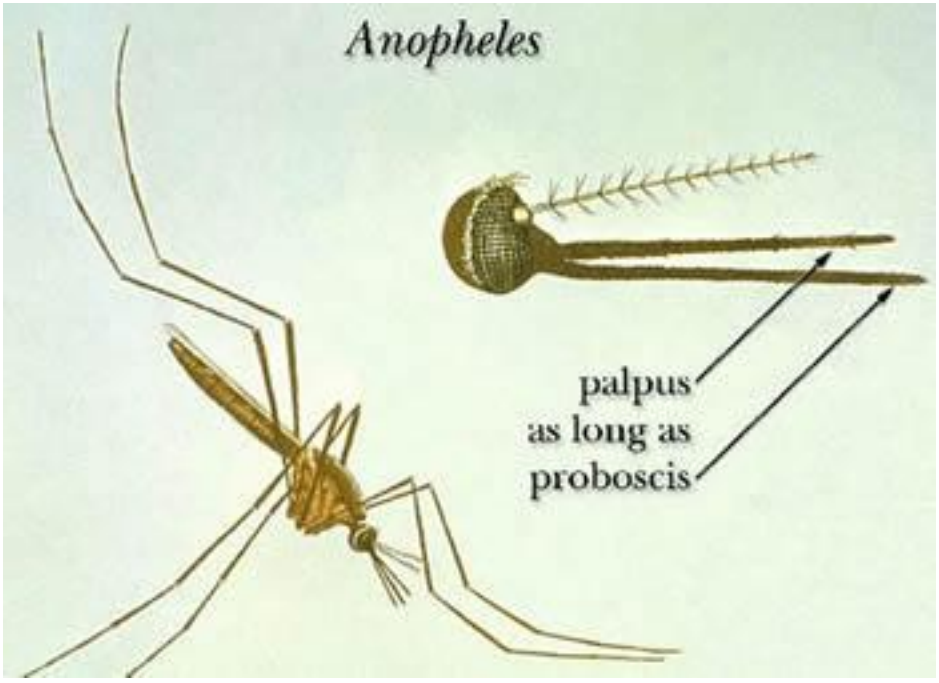


Figure 2.4: Image of an adult Anopheles mosquito

This species long palpus and proboscis, the resting position is usually at 90ºC.

Source; *https://www.cdc.gov/mosquitoes/gallery/index.html*

2.2.3: *Aedes* species

This species lay eggs in containers with collected rainwater. Aedes mosquitoes lay eggs on the interior surface of water containers. This is a survival tactics as the eggs usually have enough time to complete their developmentatal stages before the water completely evaporates (Hotez & Kamath, 2009). *Aedes* species usually bite during the day hence know vectors for diurnal sub periodic *W. bancrofti* and *B. Timori* parasite. Aedes mosquitoes are anthropophilic meaning their preferred host is humans (Wilke & Marrelli, 2015). Figure 2.5 shows an image of Aedes species and its distigushable morphological features.

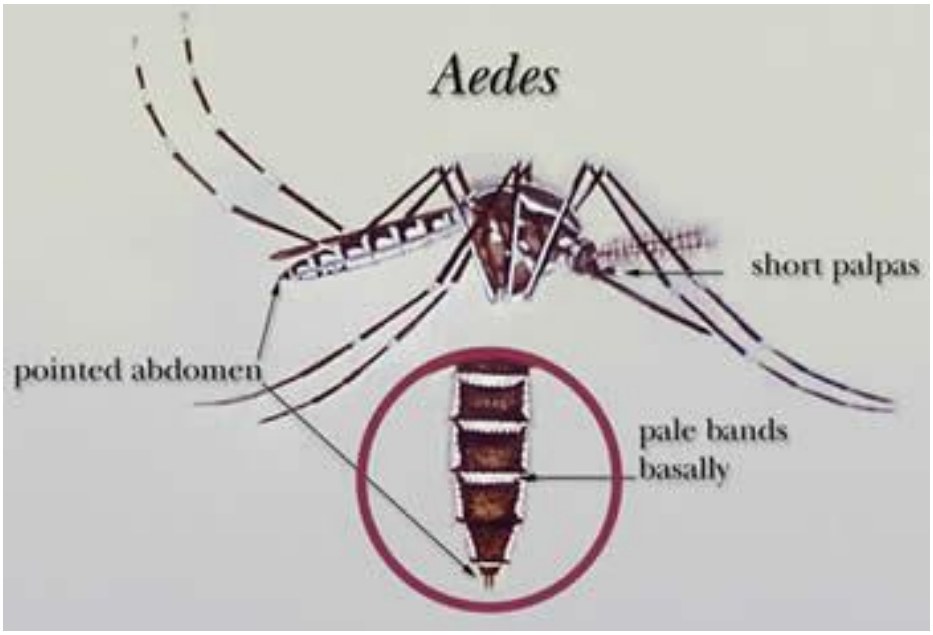


Figure 2.5: an image of an *Aedes* species of mosquito.

The tip of the abdomen is pointed with posterior spiracular bristles, the abdomen has pale basal bands, the palpas are shorter than the proboscis, it has a silver thorax with white markings.

Source; *https://www.cdc.gov/mosquitoes/gallery/index.html*

2.2.4: *Mansonia* species

This species has a characteristic large body size, with broad asymmetrical wing and have dark sparklings on the wing veins and legs. They have short palps, long proboscis and blunt abdomen. This species is found in dirty stagnant waters and are found along water mass, they are the main transmitters of *Brugian* parasites. For survival, larvae and pupae of *Mansonia* mosquitoes attach themselves on water plants on the stems or the roots and they obtain oxgen from these plants (Southgate & Bryan, 1992). *Mansonia uniformis* and *Ma. Africanus* are known brugian vectors in Asian countries, however, they have not been found to have any vectorial capacity for *W. bancrofti* though they are abundant in Kenyan endemic regions (Kinyatta *et al*., 2011). Onapa and others (2007) in Uganda, and Ughasi *et al*., (2012) in Ghana have shown that this species does not transmit *W. bancrofti* under natural condition because they are not capable of carrying larvae to infective stages of L_3. Figure 2.6 shows an adult Mansonia mosquito species with its deferential features.

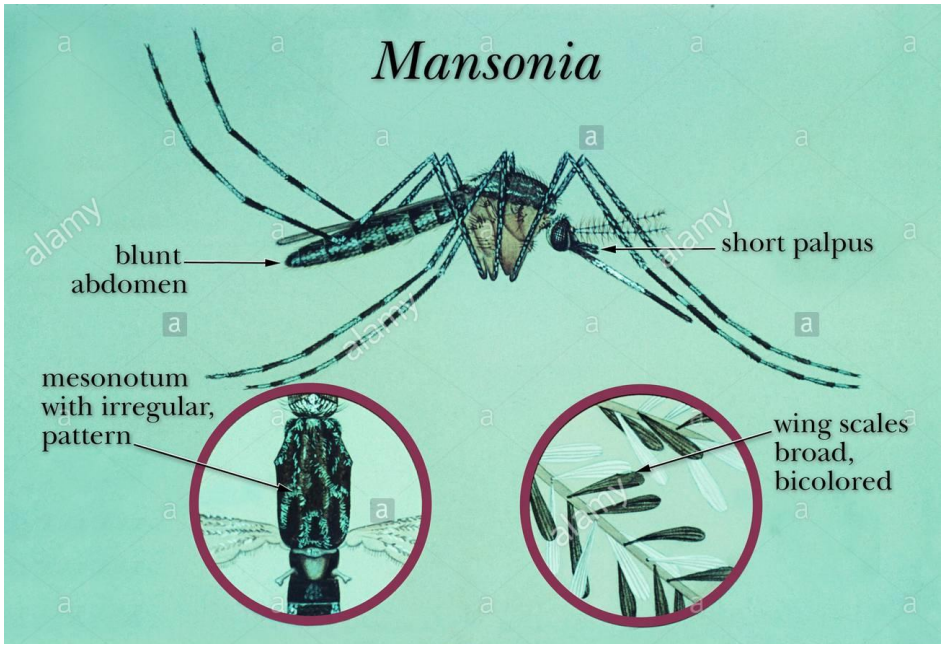


Figure 2.6: an image of a typhical adult *Mansonia* species

This species has unique broad asymmetrical scales on the wings that are dark and pale in colour. The abdomen is blunt, short palpus and the head has mesonotum with irregular patterns**. Source;** *https://www.cdc.gov/mosquitoes/gallery/index.html*

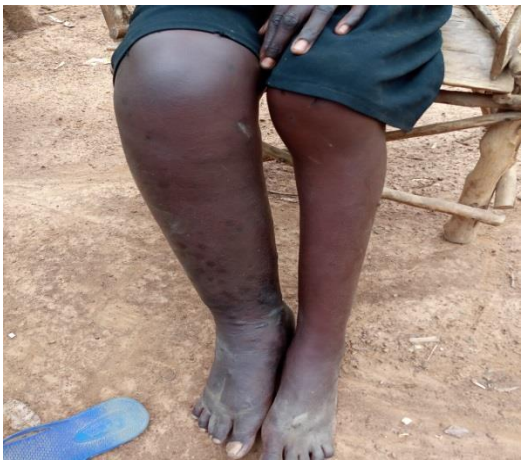
2.3: Mosquito control

Disease vector control interventions is an important strategy towards elimination mosquito borne diseases (WHO, 2013). Filariasis cause a high degree of morbidity while malaria leads to high mortality rate allover the world (WHO, 2011). Common vector control programmes for these two diseases reduces the time, cost and its easy to sustain the programmes since they are concomitantly found in similar geographical regions (Van den Berg *et al*., 2013; De Souza *et al.,* 2011). For instance, enhancing vector control measures in malaria endemic zones where there is co-infection with filariasis improves elimination precess (WHO, 2013; Trusting *et al*., 2013; Menguin *et al*., 2010). *Culex, Aedes* and *Manisonia* species can readily be controlled by environmental management such as improved sanitation, clearing bushes around homesteads, removing waste containers and space insecticide spraying to reduce vector breeding sites (Sunish *et al.,* 2007; Trusting *et al*., 2013; WHO, 2013).

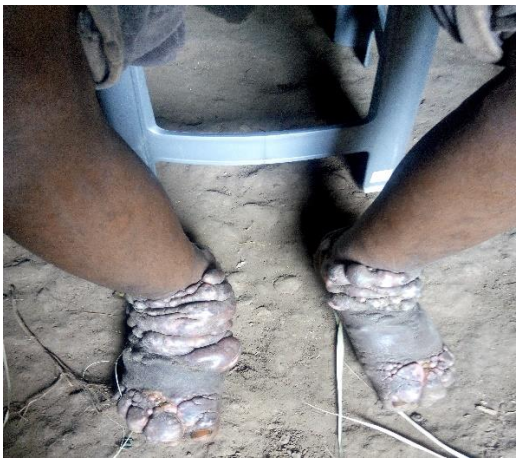
2. 4: Symptoms of lymphatic filariasis

The disease manifest as asymptomatic and symptomatic forms. Many of the asymptomatic cases have no signs but they carry microfilariae contributing to transmission and may lead to changes in the body immune system which damage the lymphatic systems and kidneys (WHO, 2020). The infections take 5-15 years to manifest to chronical stages which includes lymphoedema, elephantiasis and hydrocele (WHO, 2020). Though not fatal, there is a high level of social stigmatization in the society and loss of income including high medical cost associated with the morbidities (Martindale *et al*., 2014; Christopher *et al*., 2020). Filarial episodes of acute attacks and high fevers are due to secondary bacteria and fungal skin infections as body's defence mechanism has been compromised. The attacks

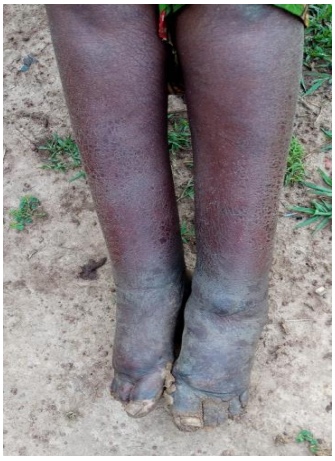
result to work and school days lost during the acute attacks (Krol *et al*., 2013). Figure 2.7 shows chronic stages of lymphedemas and hydrocele cases as found in the study areas.



a.

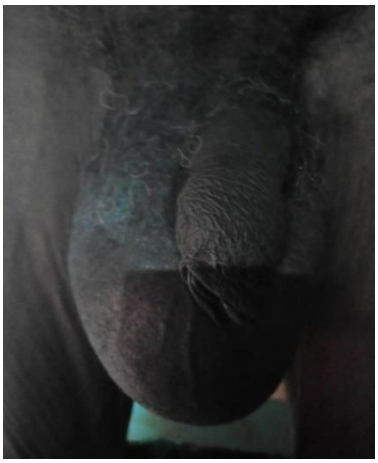


b.



c.

d.



e.

Figure 2.7: Swollen lower limbs and scrotum in male patients from Tana River Delta and Busia Counties due to lymphatic filariasis; (a) shows lymphoedema of one leg, (b, c, d) shows lyphoedeme of both legs and (e) is scrotal swellings. **Photo credit:** Nancy Kinyatta

2.5: Diagnosis of *Wuchereria bancrofti*: review of advances in helminths diagnosis

Helminth diagnosis has advanced greatly since conception of lymphatic filariasis elimination programmes. WHO road map for control of Neglected Tropical Diseases

recommends access to accurate diagnostic tools to be used in mapping and re-mapping, assessment of the control programmes, to determine when to stop MDA and carry out post elimination surveillance (WHO 2021a). Initially, immunochromatographic tests (ICT) were the main gold standard tool for diagnosis of *Wuchereria bancrofti,* because of lost sensitivity, cross-reactivity with other species and unavailability, it was replaced by Filarial Test Strips (FTS) with a higher sensitivity (Weil *et al*., 2013; Weil *et al*., 2013; Gounaue-Kamkumo *et al.,* 2015). During MDA scalling up and other control intervetions, more accurate, cost and time effective diagnostic tools are key factors in the elimination progress (McCarthy *et al.,* 2012). When low levels of infections are overlooked, disease re-occurance is more evident even after elimination levels are attained. Use of the appropriate tools will enable early disease diagnosis and embarking of control measures, this reduces the progression of the disease to acute chronic stages. Despite the great achievements by researchers in the field of diagnostics of helminths and the realization of the importance of effective and accurate diagnosis, there is lack of funding and governmental commitment in resource limited countries, thus, the programmes failed to achieve 2020 goals for NTDs elimination as it was targeted (WHO, 2021a; Hortez *et al*., 2016). Therefore, there is a need to develop new tools and also enhance the implementation of the existing tools during the scalling up of the control efforts. Development of acid-based detection tools have offered a solution on sensitivity in places of low infection rates and where large numbers need to be detected, multiplexing PCR assays have played an important role. They are also suitable in monitoring parasite genetic variations for development of diagnostics, drugs and vaccines for these parasites. However, the need for high skilled personnel, a lot of time involvement and high cost of equipments make these tools not suitable in low-income resource endemic countries (Espy *et al*., 2016; Verweij and Stensvold, 2014). Amplification methods which use constant temperatures to amplify DNA of various

pathogens are available. These isothermal methods are cheaper in terms of equipment requirements because they can use simple apparatus such as heated waterbaths and heat blocks. They are simple, user friendly and the time required for applification is shorter compared to that of PCR. Some isothermal methods have easy ways of detecting the amplicons using pH sensitive dyes. They have formed bases for suitable tools for point-of-care use in poor endemic regions (Craw and Balachandran, 2012). Isothermal LAMP assay has been applied in different pathogens including malaria parasites, schistosoma species and a number of viruses (Han, 2013; Deborggraeve and Buscher, 2012; Notomi *et al*.,2000). More isothermal methods are Nucleic Acid Sequence-Based mplification for retroviral replication, leishmaniasis parasites and trypanosomiasis (Craw and Balachandran, 2012; de-Ruiter *et al*., 2014; Mugasa *et al*., 2014). Recombinase polymerase amplification method uses recombinase enzyme to amplify DNA in 20-40 minutes (Rosser *et al.*, 2015; Faye *et al*., 2015; Craw and Balachandran, 2012; Piepenburg *et al*., 2006). Therefore, there is need to expand the use of accurate and cost-effective molecular tools in incountry laboritories and for field use in the era of fighting neglected tropical disease.

2.5.1: Microfilaria detection in blood smear and dissected mosquitoes by microscopy

Early diagnosis, treatment of the infected individuals, effective tracking of drug administration and programme monitoring and evaluation determines the success of control programmes. Detection of microfilariae by microscopy is a method that has been used largely in determining the prevalence and active transmissions (Farrar *et al*., 2013). However, microscopy effectiveness depends on the expertise of the observer, thus integrating it with antigen detection and having different observers can help in result validation. The nocturnal periodicity of microfilaria forces blood collection and surveys to be performed during the night hours (2200-0200 hours), which is often not easily acceptable by the people living in endemic areas (Gounaue-Kamkumo *et al.*, 2015; Rosen,

2009). Furthermore, the chances of getting microfilaria in the blood smears are very slim in cases where the microfilaria density in patients is less or during pre-patent stage of infection. This makes the method ineffective in diagnosis since it may provide false negative results. To increase the methods sensitivity, concentration technique is used. Collection of night blood and the concentration of microfilariae by membrane filtration improves the efficiency of microscopy detection and quantifies infection load (Kerketta *et al.*, 2012; Schultz, 1988; Schuurkamp *et al*., 1990; Sabry, 1992). Microfilariae circulate in peripheral blood at night to concide with mosquito feeding time for transmission. Once clinical signs manifest, adult worms die and no longer produce microfilaria (Mccarthy, 2000). Mosquito vectors are dissected on a slide under a microscope to search for various larvae stages of microfilariae (L_1-L_3) (Plichart, 2006; Stolk *et al*., 2004). This method is very tedious, laborious and the need for an expert in microscopy. Figure 2.8 shows a well-defined *W. bancrofti* microfilaria as seen under a microscope.

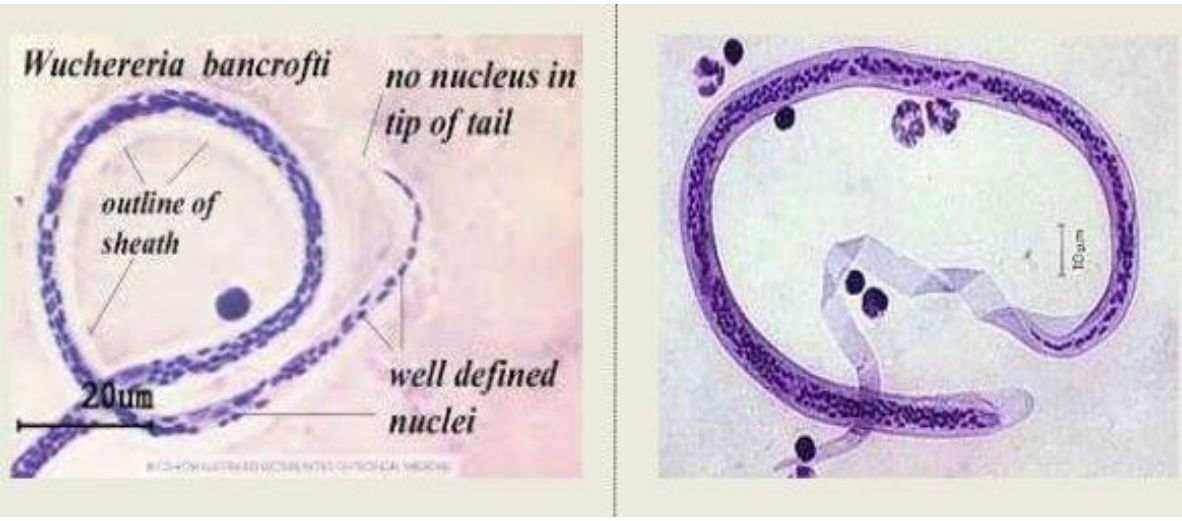


Figure 2.8: Illustration of *Wuchereria bancrofti* microfilariae as observed under a microscope

Wuchereria bancrofti microfilariae has well defined nuclei and non at the tip of the tail and this is a distiqushable feature from other microfilariae species. It has a sheath and the microfilaria take up dye on staining. Source; *https://uomustansiriyah.edu.iq/*

2.5.2: Binax Now® Filariasis test (ICT) and Alere™ Filarial Test strip (FTS)

These are rapid methods based on monoclonal detection of circulating filarial antigen. They are both useful for detetction of *Wuchereria bancrofti* antigens even with blood collected during the day time. Blood from finger prick is added slowly on test strip and kept at room temperature for and the results read within 10 minutes to avoid false positives (Rocha *et al*., 2009).

Victims of *W. bancrofti* usually have high levels of antifilarial IgG4 that can be detected by recombinant antigen-based rapid IgG. Antibody Enzyme Linked Immuno-Sorbent Assays are are used to test for Brugian infections (Rahmah *et al*., 2003; 2001; Weil *et al*., 1997). The antibody detections are more advantageous than the existing conventional methods. It can be used to diagnose the infection status amongst travelers, young children and prepatent infections (Baskar *et al*., 2004; Lammie *et al*., 2004). Peptide-based ELISA detects microfilariae specific antibody reactivity (Pandiaraja *et al*., 2010). Antigen based assays have the limitations that they can not differentiate between a current from past infection and they have cross-reactivity with other parasites which leads to false positives.

2.5.3: Ultrasonography

Adult worms in the lymphatics can be imaged using megahertz frequencies of 7.5 MHz (dreyer *et al*., 1996c). The characteristic twirling dance of the adult worms in the lymphatics is viewed by ultrasound in scrotal lymphatics and female breasts.

2.5.4: Lymphoscintigraphy

Radio- labelled albumin has been used to inject filariasis victims to view functioning of peripheral lymphatic systems (Ottesen and Campbell, 1994). The dilation and obstraction of the lymphatics are viewed and imaged by gamma camera (Shelly *et al*., 2006; McCarthy *et al.*, 2000).

2.5.5: X-ray

Tropical pulmonary eosinophilia signs are examined by X-rays to view the interstitial thickening of the increased bronchovasular markings (Ottesen, 1990 and Ottesen, 1992). During early infections, there is increased haemaetological levels of eosinophils count.

2.5.6: PCR Amplification methods

De-oxynucleic acid amplification assays have high accurancy as compared to microscopy and ICT (Fink *et al.,* 2011; Rao *et al*., 2016). Xenomonitoring by PCR is used for detection of *W. bancrofti* and *B. malayi* in vectors (Goodman *et al.*,2003; Laney *et al.*, 2008; Intapan *et al*., 2009). Infection rates in vectors have decreased drastically following control interventions. Disease transmission estimations require large numbers of mosquitoes whereby appropriate mosquito collection methods are used to maximize the catches incase of low infection rates (Kinyatta *et al*., 2018). *Wuchereria bancrofti* DNA have been isolated and amplified from whole blood, serum, sputum and urine from victims (Kagai *et al*., 2008; Abbasi *et al*., 1998; Lucena *et al*., 1996). However, PCR assays have only been laboratory based due to high cost of equipments and the requirement for specialised technical staff (Nuchprayoon, 2009). Various dot blots including northern and western blots are used in detecting biomolecules in molecular science (Abbasi *et al*., 1999), though they have not been used widely.

2.5.7: Isothermal amplification methods

Apart from thermal-cyclic DNA amplification, amplification can be done at a constant temperature by a heat block or water bath (Notomi *et al*., 2000). Loop-mediated isothermal method, nucleic acid sequence-based amplification, helicase-dependent amplification and the recombinase polymerase amplification assay have been developed for diagnosis of pathogens (Zonali and Giuseppe, 2013).

2.5.7.1: Loop- mediated isothermal amplification assay (LAMP)

This method uses four or six primers to amplify eight different regions on primer binding, with a high specificity and sensitivity that is comparable to PCR (Notomi *et al.,* 2000). Parida *et al*., (2004) developed LAMP assays for detection of RNA and DNA of West Nile while Poon *et al*., (2004) developed LAMP for SARS viruses. LAMP assays have also been developed by parasitogists for detection of human parasites including *W. bancrofti* (Kinyatta *et al*., 2021; Takagi *et al*., 2011), *S. Mansoni* (Ndungu *et al*., 2018), *Entamoeba* (Lianga *et al*.,2009), *Trypanosoma* (Njiru *et al.,* 2008), Taenia (Nkouawa *et al*.,2003), *Plasmodium* (Poon *et al*., 2006), and *Cryptosporidium* (Bakheit *et al*., 2008) and for detection of *Plasmodium* and *Dirofilaria immitis* parasites in vectors (Aonuma *et al.,* 2008). All these studies have reported greater advantagies of LAMP over PCR as the method is simple, can complete the run within short time and it requires low cost equipments. More importantly, the by-product for LAMP can easily visualization by naked eyes or pH sensitive dyes without the need for running an electrophoresis gel (Mori *et al*., 2001). LAMP assays have a high potential of being used in the field set up and thus most applicable in low-income resource countries.

2.5.7.2: Recombinase polymerase amplification (RPA)

This is a one tube lyophilised reagents that can detect RNA or DNA of pathogens. The method works at a constant temperature between 37^{o}C-42^{o}C by use of reverse transcriptase enzyme (Piepenburg *et al*., 2006; Amer *et al*., 2013; Abd EL Wahed *et al*., 2013). The method can amplify DNA at room temperature but at a slower rate, this makes the method more superior to other isothermal methods and thus more suitable for field set ups (Escadafal *et al.,* 2013). A few copies can be amplified to detectable levels within 5-10 minutes (Euler *et al*., 2013a; Amer *et al*., 2013)

2.5.7.3: Helicase-dependent amplification (HDA)

Double stranded DNA is separated by helicase enzyme for the purpose of formation of new strands when the primers bind (Vincent *et al*., 2004; An *et al*., 2005). There is exponential DNA amplification occurs at a constant temperature without requirement of thermal cycler (Eisenstein, 2004). Amplicons are detected by real-time or gel electrophoresis (Gill *et al.*, 2007a; b; c). Use of crude samples for amplification has been demonstrated by studies by Ghedin *et al*., (2004).

CHAPTER THREE: MATERIALS AND METHODS

3.1: Study site

Human blood specimens and mosquitoes were collected from Tana River Delta in Tana River County, Malindi and Matayos Constituency in Busia County (Figure 3.1 and 3.2 respectively). According to studies by Kagai *et al*., (2008), Tana River Delta region was found to have high initial antigenaemia prevalence of (22.2%) before the start of MDA Programmes in the region in 2011 (Njenga *et al*., 2017). Tana River County has a total area of 37,951Km^2 and a population of 315,943 translating to a population density of 8.325/Km^2 as per the population census, 2019 (Kenya National Bureau of statistics, 2019). The Delta, has three constituencies; Garsen, Galole and Bura occupying an area of 16,013Km^2. The region temperature range is 30^{o}C with an altitude range of 0-200m and receives erratic rainfall ranging between 220-900mm per year causing floods. The economic activities practiced in this region are farming, fishing and livestock keeping. Pokomo, Wardey and Orma are the main ethic groups in this region.

Mpirani village in Malindi constituency Kilifi County had a high prevalences during initial mapping (Wamae *et al*., 2001). MDA in Malindi begun in 2003 (Njomo *et al*., 2004). Malindi town is 120 Kilometres Northeast of Mombasa and cover a geographical area of 7,605 square Kilometers. The constituency of malindi has a total population of 119,856 as per 2019 census (Kenya National bureal of statistics, 2020). The main industry is tourist attraction and fishing. The region has temperatures above 25^{o}C and receives two rain seasons of 800-1000mm per year. Human economic activities and environmental conditions favour mosquito breeding for *W. bancrofti* transmission in these regions (Kinyatta *et al*., 2018).

Matayos South and Busibwabo wards in Matayos constituency, Busia County were selected for the study due to filarial-like lymphedemas previously reported (Kinyatta,

unpublished data), however, there are no available data establishing its endemicity. The region has ecological factors which favour mosquito breeding for vector borne disease transmission. Busia County (00 27 11N, 34 07 30E) is located in Western part of Kenya, approximately 268 miles (431 km) west of Nairobi and East of Busia district in Uganda. The 2019 population census estimated the population of Busia to be 816,452 (Kenya National Bureau of statistics, 2019). The county covers an area of 1,700Km^2 and the main economic activities are fishing and agriculture. The mean annual rainful range is 900mm-1,500mm with temperature range of 17^{o}C -30^{o}C. It has two rain seasons with long rains falling between March and June and the short rains between September and October.

Figure 3.1 shows study villages of Tana River and Malindi in Coastal Kenya and figure 3.2 shows study villages of Matayos and Busibwabo in Western Kenya.

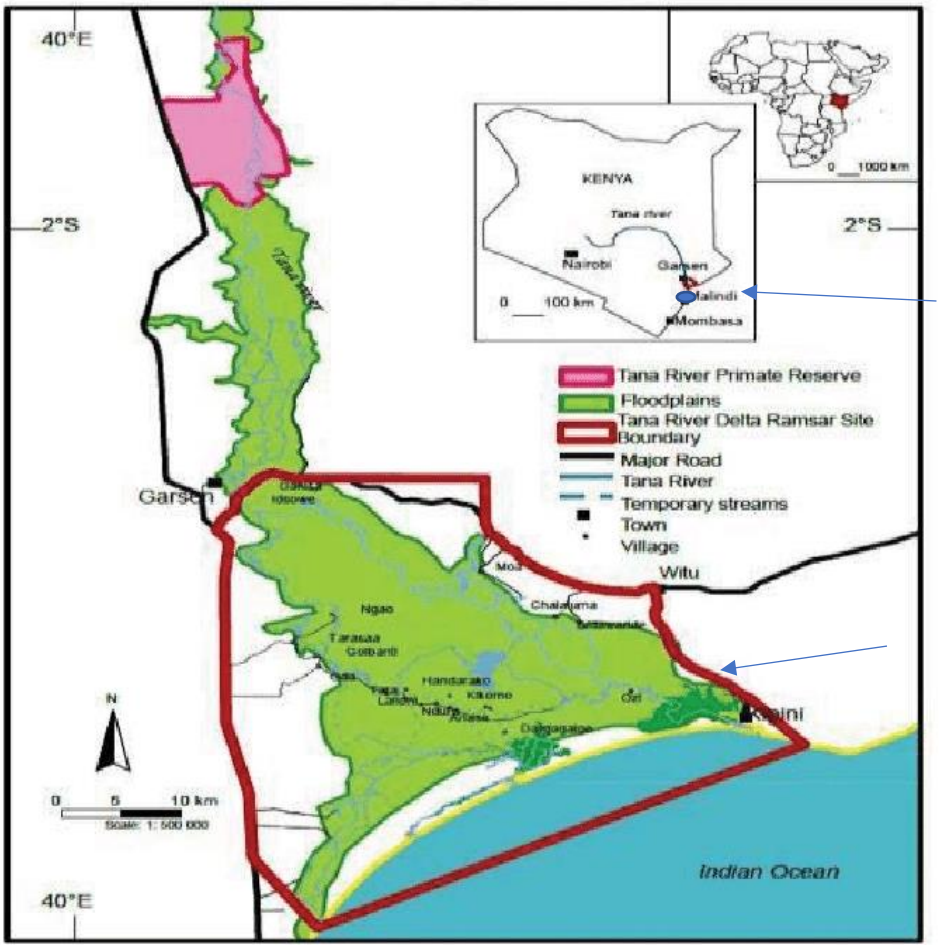


Figure 3.1: A map of Tana River Delta and Malindi in Kenya

Study villages in Garsen and Kipini municipalities in Tana River Delta are enclosed by red line. Study villages in Malindi is encircled in blue and pointed by a blue arrow on the map of Kenya. **Source;** https://www.wetlands.org/blog/photo-kenyas-tana-river-delta-ramsar-sitelaunched/

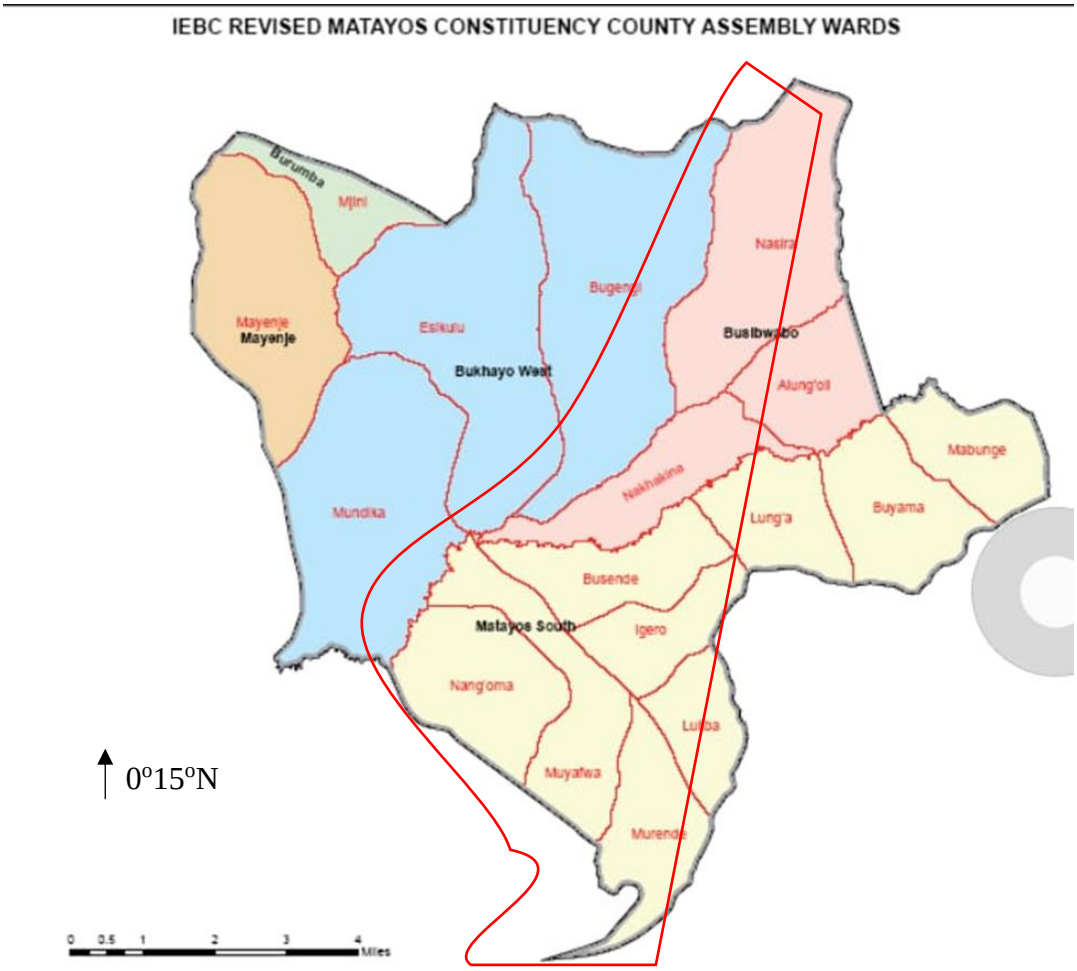


Figure 3.2: A map of Matayos constituency wards in Busia County, Kenya

Villages in the study sites of Matayos south and Busibwabo wards enclosed in blue line

Source; *https://matayos.ngcdf.go.ke/*

3.2: Study population and mosquito collection

Hundrend serum samples were selected on the bases of ICT and PCR positivity from archieved samples from Tana River Delta and Malindi for genetic characterization objective in this study. For assay optimization and validation assays, blood samples were collected from. Participants were recruited first by filling a consent form for blood specimen collection. In-door and out-door resting mosquitoes were trapped using CDC light traps powered by rechargeable batteries from randomly selected participant's houses who turned positive for circulating filarial antigens by FTS.

3.2.1: Population selection criteria and sample size calculations for blood collection for LAMP assay development and validations

Participants selection criteria were based on disease chronic signs and those of the victim's households. Persons above 2 years of age and who have lived in the study area for over 2 years were legible. Participation was on voluntarily basis and the participants were requested to consent for the adults and give assent for their children.

Sample size was given by the formula of Fischer *et al*., (1998) as;

Formula: $\mathbf{N= Z^2 P Q /d^2}$

whereby; d = The minimum expected error (0.05),

N was minimum sample size, Z was standard (1.96), P was prevalence within target population, which is estimated at (22 %) and Q is 1 – (prevalence)

Calculation;

$N = (1.96)^2 x\ 0.22\ X0.78 = 264$ (Human participants)

$\mathbf{d^2} = (0.05)^2$

A minimum of 264 participants were needed in both study sites. 387 participants were recruited. Busia County had a total of 262 and Tana River County had 125 participants.

3. 2 .2: Mosquitoes sample size for assays validations

Since the population size of mosquitoes in the study areas was not known and is generally difficult to estimate in any site, the non-finite approach to sample size determination by the Specified Precision Estimation (SPE) method (Louangrath, 2014) was used to calculate the minimum number of houses targeted for mosquitoes sampling.

The estimate by SPE method is given by the formula;

Formula $\mathbf{n = [Z0.95\sigma N\ (0,1)]/E}$

where Z0.95 = standard score for 0.95 Confidence Interval = 1.96

σN (0,1) = population standard deviation for standard normal distribution= 1

E = Minimum expected error = 0.05

n = 1.65(1)/0.05 = 33

Thus, a minimum of 33 houses were targeted for mosquito collection from Tana River and Busia, Counties. Mosquitoes were collected in 40 houses in Tana River and in 51 houses in Busia.

3.3: Sample collection

3.3.1: Human blood sampling

Four (4ml) patients' blood was drawn aseptically using venipuncture of the median cubital vein in well-labelled EDTA vacutainers during the day. The blood was let to stand for 2 hours to separate serum from cell and the sera was put in a well labelled 2ml vials. Blood samples were packe in cooler boxes containing ice packs and transported to KEMRI, Centre for Biotechnology Research and Development laboratories. All participants together with other family members were treated with Albendazole. Figure 3.3 shows a clinician in Garsen bleeding a patient in a field set up.

Figure 3.3: A clinician bleeding a patient during blood collection exercise in Garsen, Tana River Delta

Photo Source: Nancy Kinyatta

3.3.2: Mosquito collection

Battery powered CDC Light traps with CO_2 baits (John W. Hock. Co. Florida, USA) were used for mosquito collection as shown in figure 3.4 below. The traps were set between 6.00PM to 6.00AM in participants' houses in the bedrooms/ sleeping area to maximize the chances of getting fed infected mosquitoes. At the same time, out-doors traps were set at doorsteps or at the windows and also near the cow sheds to capture mosquitoes entering the houses for blood meal. Mosquitoes were killed by exposing them to chloroform and identified according to taxonomic key (Rattanarithikul, 2005a). The mosquitoes were pooled into approximately 20 mosquitoes and preserved under silica gel for DNA extraction and amplification as per the procedure by Takagi *et al*., (2011). Figure 3.4 is CDC light trap being assembled for mosquito trapping.

Figure 3.4: Image demonstrating how to set CDC light traps for mosquito collection

3.4: Sample processing

3.4.1: Alere™ Filariasis Test Strip

Circulating filarial antigen test was carried on FTS kit (Scarborough, ME, United states). The card and the plastic work tray were removed from the foil pouch package. A sticker labeled with patients details or a coded number was used to firmly hold the strip on the working tray. Seventy-five microliters (75µl) of blood specimen were slowly added to the lower part of the sample pad. The results were read and recorded within 10 minutes. Positive specimen showed pink lines on control and test positions while negative specimen shows only control line. Results without bands or without the control line were regarded as void and the test was repeated.

3.4.2: DNA extraction from serum samples for amplification Assays

Two hubdrend microliters of serum samples were aliquated into single well labelled eppendorf tube and mixed with 250µl of sodium hydroxide (NaOH) containing 1% Triton. The tubes were placed on a thermomixer heated at 65ºC for 30 minutes and the pH was determined by pH- meter and adjusted to pH 8 by adding a drop of 1N hydrochloric acid or 1N sodium hydroxide depending on the alkalinity or acidity of the mixture respectively. After spinning, the mixture at 14000 RPM at 4ºC for 5 minutes, supernatants were transferred into clean eppendorf tubes. Eight hundred microliters of absolute ethanol (99.9%) were added and incubated at -70ºC freezer for 1-12 hours. After incubation, the mixture was spanned for 20 minutes at 4ºC, 14000 RPM and the supernatant discarded. The decant was washed thrice with 70 % of ethanol and dried in open air for 30 minutes. 50µl of TE buffer was added to dissolve the DNA, vortexed and stored at -20ºC. A known positive control and negative controls were included during the DNA extraction process to validate the extraction method.

3.4.3: DNA extraction from mosquito samples

A pool of one to twenty (1- 20) mosquitoes of the same species were put in 1.5ML tubes and ground in 100µl of grinding buffer, DNA extraction was done as by Fischer *et al*., 2003. The tubes were heated at 65ºC in thermos mixer for 30 minutes. Fouteen microliters of 8M potassium acetate was added and vortexed and the mixture centrifuged at 4ºC, 14000 RPM for 10 minutes. The supernatant was pipetted out into clean labeled Eppendorf tubes. Two hundred microliter (200µl) of absolute ethanol (99.9%) was added and vortexed for 1 minute then incubated at -70ºC for 2 hours or overnight. After incubation, the mixture was spun at 4ºC, 15000 RPM for 20 minutes and supernatant discarded. The remaining mixture was washed three times using 70% ethanol and air dried for 30 minutes. The DNA was

dissolved in 50µl TE buffer, voltexed and stored at -20 ºC freezer for use during amplification.

3.4.4: Conventional PCR assay and gel electrophoresis

PCR assay for DNA amplification was done using species specific primers NV1 and NV2 to amplify a Ssp1 repeat sequence of 188 base pairs in *W. bancrofti* (Zhong *et al*., (2006). Forward Primer (NVI) sequence was 5'-CGTGATGGCATCAAAGTAGCG-3' and that of reverse primer (NV2) was 5'- CCCTCACTTACCATAAGACAAC-3' at 5pmol concentration (sigma-Proligo, Singapore). A nanodrop ND-1000 spectrophotometer (ThermoScientific, USA) was used to measure DNA concentration given by A260 and a ratio of A260/A280 on a Nanodrop ND-1000 spectrophotometer (ThermoScientific, USA). The PCR reaction volume of 50µl consisted of a PCR Master mix containing; 10X PCR Buffer (5µl), 0.5µl each primer, 100mM dNTPs mix (0.5µl) and PCR water (38.25µl). Master Mix was aliquoted in PCR vials and 5µl of template and 0.25µl of Taq DNA polymerase added. The vials were incubated in Gene Amp® PCR system (Applied Bio system, Singapore 9700) for 5 minutes at 95ºC. This was run for 35 cycles of denaturation, annealing and extension programmed at 95ºC for 30 seconds, 54ºC for 1 minute and 72ºC, 30 seconds respectively. Last extension for 10 minutes at 72ºC was allowed before the reaction vials were removed for amplicon detection. PCR product size fragmentation was run on 2.0% agarose gel electrophoresis and the product read at 188 base pairs against a 100bp molecular weight marker.

3.4.5: Loop mediated isothermal amplification assay optimization

Assay by Takagi *et al*., (2011) for amplification of *W. bancrofti* 18S rRNA fragment in human and vector mosquitoes were optimized and validated. The designed primers by

Takagi *et al*., targeted a gene with more copies and less variants in the repeat sequence. The primers had been designed by ExplorerExplorer Version 4 Software (https://primereplore.jp/e/v4_manual/pdf/PrimerExplorerV4_Manual_1.pdf). Two sets of external primers, forward and backward and forward and backward internal primers were used. For optimization, the reaction master mix contained 25µl of 0.2µl of *Bacillus stearothermophilus* polymerase, (New England Biolabs), 1.6 µM of FIP and BIP each, 0.2 µM of F3 and B3 each, 1.4 mM dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM (NH4)2SO4, 8 mM MgSO4, 0.1% Tween-20 and 5µl of extracted DNA from human whole blood or mosquito pools. The reaction was incubated at 63°C in a water bath for 60 minutes. The amplified DNA was visualized in the form of colour change when SYBR Green I or a florescent dsDNA intercalating dye was added. The amplicons were fragmented on 2% agarose gel stained with ethidium bromide and viewed using UV light at 320nm.

3.4.6: Helicase dependent amplification assay

This amplification reaction contained helicase enzyme (100ng UvrD helicase), 10 × HDA buffer containing 50 mM Tris-acetate (pH 7.5) and 100 mM dithiothreitol and NaCl. Ten microliters (10 µl) of buffer were added to 2.5 µl of DNA exteact from human blood sample in a reaction tube in addition to 0.5 µl forward and 0.5 µl backward primers, 0.5 µl of polymerase enzyme, 2 µl of 20 nmol dNTPs and dH_2O to top up the reaction to 25 µl. The reaction components were incubated at 60^oC for 2 hours. Product size fragmention was done by running the amplicons on 2% agarose gel electrophoresis with ethidium bromide. Optimization was done with varying volumes of the reagents and other parameters including temperature ranging from 60^oC to 65^oC and time between 30 minutes to 60 minutes.

3.5: Validity and reliability of the isothermal amplification methods

3.5.1: Evaluation of assay sensitivity

DNA extracts from positive samples were diluted into 10-fold serial dilutions (10^0-10^9) and each level amplified by LAMP, PCR and HDA assays. The products were detected on agarose gel to evaluate the concentration level at which each method was sensitive. The sensitivities were analyzed and compared against conventional PCR as the standard method.

3.5.2: Evaluation of assay specificity

LAMP and HDA assays specificity was done by detection of DNA extracts isolated from other parasites; *Brugia malayi*, *Schistosoma mansoni*, *Plasmodium falciparum*, *Trichuris trichiura, Leishmania donovani*, *Echinococcus granulosus,* and *Anopheles gambiae* species. The integrity of the experiment was compared to PCR including negative and positive controls. Positive control used was generously provided to KEMRI Filariasis Research Laboratory by Hebrew university, Israel while PCR water and blood sample from filarial non-endemic area were used to validate the results.

3. 5.3: Clinical determination of the assay accuracy

Calculation of the accuracy of LAMP and HDA assays were determined by the specificity, sensitivity and the predictive values of each method against PCR and gel electrophoresis as the standard.

Sensitivity; True positive/True positive + False positive

Specificity; True Negative /False positive +True Negative

Positive predictive value; True positive/ True positive + False positive

Negative predictive value; True Negative/True Positive + False Negative

3.6: Field evaluation of isothermal amplification methods

The 1sothermal assay found to have superior accuracy was used in the field validation to assess their applicability as point of care diagnosis of the subjects as described in chapter 6.

3.7: Data management

Participants were requested to consent before participating. Subject bio-data was filled in bio-data forms and recorded in data book and Excel spreadsheet software. Participants' details were maintained confidential, and codes known only to the PI were used in entering the data in spreadsheet software. The results of each participant were communicated directly and confidentially to each subject. All records were kept in lock and key cabinet and zipped documents in computer folders.

3.8: Statistical data analysis

Genetic data was analyzed by clustalW, MEGA version 7 software and Basic Local Alignment Search Tool (BLASTn) to determine genetic variations in parasites population. The degree of variation in DNA sequences and the statistical significance of the methods was determined at a 95% Confidence interval. Statistical data analysis for sample analysis was done by statistical software version 23.0. Participants and mosquitoes' descriptive statistics were determined. Contingency tables 2x2 were used to compared the sensitivity and specificity of LAMP and HDA against cPCR, Kappa statistics was determined to show the level of agreement between the methods. Conventional PCR was regarded as "gold standard" method in the analysis to evaluate the accurancy of LAMP and HDA isothermal amplification assays. Sensitivity of the test results is evaluated as the percentage of positive results by the test to that of the standard method used while negative results gave the specificity of the test under evaluation.

3.9: Ethical Considerations

The approvals for the study were sought from; scientific steering committee and Ethical Review Unit of Kenya Medical Research Institute, University of Nairobi board of Postgraduate studies and Ministry of Health authorities in the two study Counties. The participants consented for the study willingily after the purpose of the study was explained to them in details. Blood was drawn either by finger prick or vein puncture from the participants who gave written consent. The participants were informed of minimal acceptable pain caused by needle pricks during blood collection and that they would not be losses or major risks involved in the study. There was no compensation given to the participants but the study community was given free Albendazole drugs.

CHAPTER FOUR: GENETIC CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF *WUCHERERIA BANCROFTI* 18S rRNA FOR DIAGNOSTIC TARGETS IN MALINDI AND TANA RIVER DELTA, COASTAL-KENYA

4.1: Abstract

Introduction; Development of diagnostic tools, drugs and vaccines against parasites target a highly reapted species specific gene in a sequence. Genetic information among helminths causing lymphatic filariasis is of importance in understanding the biology of the parasites and has practical implications in the control programs. Prolonged use of antihemintic drugs may cause changes in population structure of the parasite which adversely affect the tools used for diagnosis and drug interventions altering management programs. This leads to potential resurgence of resistance strains after transmission is stopped, therefore, it is of much importance to have updated information on filarial genetic structure for the control programmes to make informed and right decisions. Despite the fact that Kenya has had it's National lymphatic filariasis control program since 2002, elimination target has not been achieved. *Wuchereria bancrofti* genetic data in Kenya is lacking, therefore this study was conceived with the aim of characterizing and analysing 18S rRNA partial gene of *W. bancrofti* parasites in Kenyan endemic regions.

Methodology; Archieved human blood specimen collected from 100 individuals in Mpirani village in Malindi and Kipini villages in Tana River Delta were used in this objective. DNA extraction from serum specimens was done, *W. bancrofti* 18S rRNA marker was amplified and detected by agarose gel electrophoresis. 17 specimens out of the 100 tested were positive for *W. bancrofti* DNA. The products were purified, quantified and sequenced by Sanger sequencing. The resulting sequences were aligned and edited by Chromas version 2.6.5 and BioEdits softwares. Using MEGA version 7, 14 sequences with

good alignment were selected for analysis by determining the intra and interpopulations relationships, pair wise distance and phylogenetic tree construction.

Results; Isolates of Mpirani in Malindi and Kipini in Tana River Delta had a mean distance of 0.99 and nucleotide diversity (π) of 0.603. Nucleotide similarity was between 79 and 98% with related species in the GenBank.

Conclusion: *Wuchereria bancrofti* 18S rRNA gene sequenced was higly conserved among the 14 sequences analysed and suitable for molecular diagnostics targets with high identity similarity of 98% with *W. bancrofti* assembly of accession numbers LM012589.1, L20344 and AY297458.1

Recommendation: More genetic studies in other endemic regions need to be carried out to establish the extent of genetic diversity in endemic regions.

4.2: Genomic structure of *W. bancrofti*

Diagnosis of filariasis is based on DNA detection of a highly repeated gene, filarial antigens and ant-filarial antibodies (WHO, 2017b; Small *et al*., 2014). Control programs are yet to consinder molecular typing and genomics for determining infections evolution history (Small *et al.,* 2014). Studies on whole genomes, genotyping, target sequencing and random amplified polymophic have brought insights on the parasites' biology, diagnostics and drug targets and reviewed drug resistance strains among parasites (Small *et al.,* 2019; Ramasamy, 2011). PCR- based assays are used to identify sequence variations in related species and to track the geneology of these parasites (Hedtke *et al.,* 2019). Genomic studies assist elimination processes of most of helminthes such as *W. bancrofti.*

Wuchereria bancrofti mitochondria (mt) has a length of 13,637 nucleotides with 2 ribosomal RNAs (rrns) in the whole genome. The transfer RNAs (trns) are 22 and with 12 Protein- coding genes, it has an AT content of 74.6% (Ramesh *et al*., 2012). The size length was determined by total lengths of all scaffolds which were 81.51 Mbp, GC content of 29.70% with protein-coding genes given as 19 327. The Genome has also 112 predicted t-

RNAs genes and 8 predicted rRNA genes. (Broad Institute, 2010). Nematodes have complex life-cycle and transmissions involving an intermediate and a definitive host and have a large population size associated with high genetic variations (Criscione *et al*., 2005; Hawdon *et al*., 2001). Estimation of nuclear variations can be done on partial sequencing of a highly repeated loci other than the whole genome. They are useful in identifying effects of selection on population structure, size and migrations (Weigand and leese, 2018; Small *et al*., 2014). Other filarial mitochondrial genomes for buiding different markers of other filarial parasites are available (Small *et al*., 2012; Unnasch and Williams, 2000; Higazi *et al.*, 2004).

Mitochondria gene order of *W. bancrofti* is similar to that of *D. immitis*, *S. digitata, O. volvulus* and *B. Malayi*. Parasites' responses to drugs and identification of resistant strains are only possible by monitoring variations in their genetic make ups (Volkman *et al*., 2012). Genetic similarities among parasites are an indication of historical origin or gene flow through interbreeding. Human and vector movements have resulted to introduction of new strains of parasites or new transmissions occurring in non endemic regions (Ramaiah, 2013; Xu *et al*., 2018b).

There is scanty or even non existing data on *W. bancrofti* genetic characterization in endemic regions of Kenya. This study therefore, sequenced and analysed *W. bancrofti* 18S rRNA partial gene. This information helps with more understanding of the genetic variations and phylogenetic relationships of *W. bancrofti* 18S rRNA gene sequences from Kenyan isolates.

This study characterized 18S rRNA *W. bancrofti* partial gene of 17 Kenyan isolates, after trimming and alignment 14 sequenses, 6 from Tana River isolates and 8 from Malindi isolates were used for phylogenetic tree constraction to determine the genetic relationships

of parasite isolates from Mpirani-Malindi and Kipini-Tana River Delta, endemic study area.

4.3: Materials and methods

4.3.1: Study design

This was a longitudinal retrospective study, whereby serum specimens were selected from archived samples. Here, human subjects were not directly involved since it was based on other previous epidemiological surveillance specimens (Kagai *et al.,* 2008; Kinyatta *et al*., 2011).

4.3.2: Blood samples

Human serum samples previously collected from Mpirani-Malindi in the years 2002, 2004 and 2011 were analysed. Samples of year 2002 were collected before the start of MDA while those of 2004 were after one round of MDA and of 2011 were after several rounds of MDA. Kipini-Tana River specimen used were those collected in the year 2008 before start of MDA and in 2011 after the first round of MDA.

4. 3.3: Laboratory analyses

Specimens were analysed by extracting DNA and amplification of *W. bancrofti* 18S rRNA gene by cPCR as detailed in chapter 3.

4.3.4: Polymerase chain reaction, product purification, quantification and sequencing

Wuchereria bancrofti DNA was amplified in a total premix reaction volume of 100µl. Product purification was done using QIAquick protocol spin columns in a micro centrifuge and quality and quantity of the concentration analysed using Nanodrop 2000 (Thermo Fischer Scientific). Concentration of 1µl of amplified DNA ranged between 90.2ng/µl and 30.0 ng/µl. Amplicons with concentrations higher than 50ng/µl were adjusted by addition

of buffer to 50ng/µl while those with lower concentrations were kept for different analyses apart from sequencing. Two microliters (2µl) of the concentrated DNA were confirmed on gel electrophoresis, 5 µl of each of 17 selected amplicons were sent for Sanger sequencing (Macrogen-Europe) including 2 sets of primers used for amplification.

4.3.5: 18S rRNA *W. bancrofti* sequences analysis

Chromas software Version 2.6.5 was used for assembling the sequences which were then trimmed using BioEdit Programme. Alignment of the sequences was done on clustalW by MEGA version 7 and short sequences were excluded from further analysis. Sequences from 14 specimens met creteria for sequence analysis. Sequences related to *W. bancrofti* were obtained from GenBank nucleotide database using Basic Local Alignment Search Tool for homology comparison. Phylogenetic tree was constructed, and evolutionary distance matrix calculated.

4.4: Results

4.4. 1: *Wuchereria bancrofti* 18S rRNA amplified product detection on agarose gel electrophoresis

The *W. bancrofti* 18S rRNA gene Ssp1 repeat sequence has an expected size of 188 base pairs. Figure 4.1 shows amplicon size fragmentation on gel electrophoresis of the *W. bancrofti* DNA Ssp1 repeat sequence.

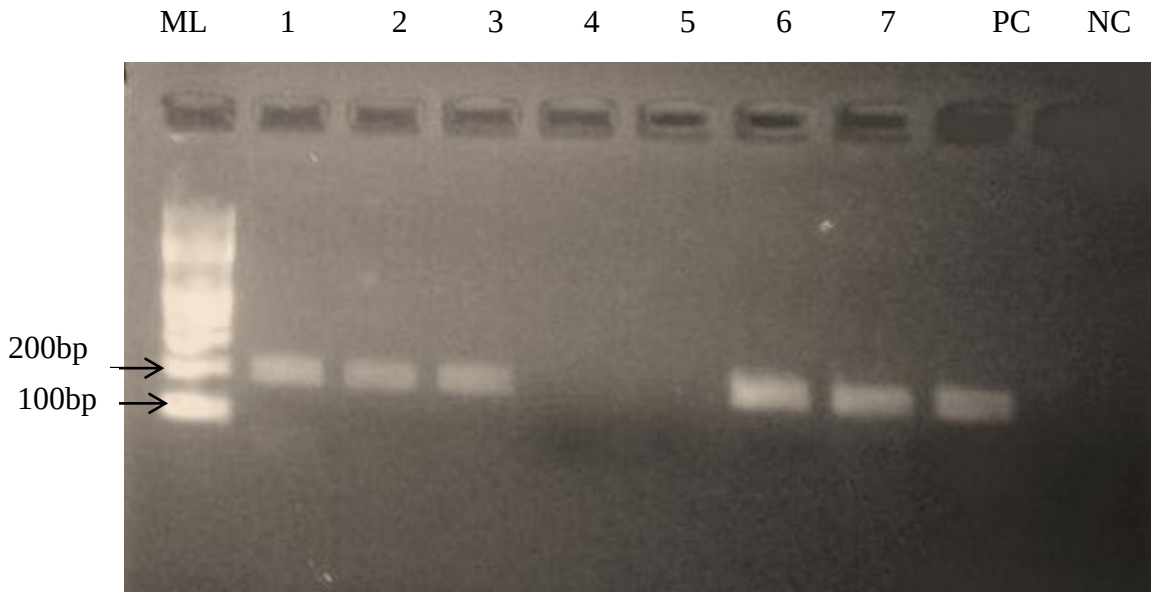


Figure 4 .1: *Wuchereria bancrofti* 18S rRNA PCR amplified product of isolates from Mpirani- Malindi and Kipini-Tana River Delta

Wuchereria bancrofti 18S rRNA amplified product size is 188bp and the bands align close to 200bp band an indication of presence of the amplified targeted gene. ML represents the 100bp molecular ladder, Lane 1, 2, 3, 6, 7 are positive specimen, Lane 4 and 5 are negative specimen, lane PC is *W. bancrofti* DNA positive control and lane NC is Negative control

4.4.2: *W. bancrofti* 18S rRNA fragment sequence analysis

Sequences from 14 specimens were assembled and trimmed for further analysis. GenBank accession numbers allocated to the nucleotide's sequences in this study were as in Table 4.1.

Out of the 14 sequences send to NCBI for accession number allocation, only 10 accession numbers were allocated as shown in the table below.

Table 4. 1: ***Wuchereria bancrofti*** **18S rRNA sequences Accession Numbers of isolates of Mpirani -Malindi and Kipini-Tana River Delta deposited in GenBank**

Number	Sequence ID	Accession Numbers
1	ML1-2002	MK471341
2	ML2-2002	MK471342
3	ML3-2002	MK471343
4	ML4-2002	MK471344
5	ML5-2011	MK471345
6	ML9-2011	MK471346
7	TR3-2011	MK471347
8	TR4-2011	MK471348
9	TR5-2008	MK471349
10	TR6-2011	MK471350

Sequences of 6 isolates in numbers 1 to 6 were from Mpirani-Malindi denoted as ML and 4 isolates in numbers 7-10 were from Kipini-Tana River Delta denoted as TR. Accession numbers allocated in GenBank were labled MK.

Blast analysis on NCBI were as shown in Table 4. 2, most of the sequences from this study were related to reference sequencesin GenBank with accession numbers; LM012589.1, LM000927.1, AY297458.1, L20344.1, AP017705.1 () with identity range of 79-98%.

Table 4.2: GenBank blast results of Mpirani-Malindi and Kipini-Tana River Delta Isolates

Sample ID	Gene description	E-Value	Identity %	Accession No.
ML1	*W. bancrofti* genomic assembly *W. b* Jakarra Scaffold WBA contig 0011217	8e-44	98	LM012589.1
ML3	*W. bancrofti* genomic assembly W.b Jakarta Scaffold Jakarta WBA contig 0011217	8e-44	79	LM012589.1
	W. b genomic assembly W. b Jakarta Scaffold WBA contig 0000579	3e-71	92	LM012589.1
	W. bancrofti nuclear scaffold/matrix, attached region.	3e-71	92	AY297458.1
ML4	*W. bancrofti* genomic assembly W.b Jakarta Scaffold WBA contig 0011217	5e-34	95	LM012589.1
	W. bancrofti nuclear scaffold/matrix, attached region	1e-143	82	AY297458.1
ML5	*W. bancrofti* genomic assembly W. b Jakarta Scaffold WBA contig 0011217	1e-05	85	LM012589.1
	W. bancrofti nuclear Scaffold/ matrix, attaced region	4e-36	83	AY297458.1
ML11	*W. bancrofti* Ssp1 repeat DNA sequence	1e-58	98	L20344.1
	W. bancrofti nuclear Scaffold/matrix, attached region	4e-58	98	AY297458.1
	W. bancrofti mitochondrial DNA complete sequences	2e-17	87	AP017705.1
TR1	*W. bancrofti* nuclear Scaffold/matrix, attached region	1e-48	93	AY297458.1
	W. bancrofti Ssp 1 repeat DNA sequences	5e-47	94	L20344.1
TR4	*W. bancrofti* nuclear Scaffold/matrix, attached region	1e-45	93	AY297458.1
	W. bancrofti Ssp 1 repeat DNA sequence	6e-46	94	L20344.1
TR6	*W. bancrofti* nuclear Scaffold/matrix, attached region	2e-52	92	AY297458.1
	W. bancrofti Ssp 1 repeat DNA sequence	4e-48	91	L20344.1

ML-Malindi Isolates, TR-Tana River Isolates on blast showing identity range of 79-98% with related sequences in the GenBank

4.4.3: Evolutionary divergence of Mpirani-Malindi and Kipini-TanaRiver Delta Isolates over different time

Figure 4.2 shows evolutionary divergence of Malindi and Tana River sequences with within mean evolutionally variations of 0.94 and 0.35 respectively. Entire population mean for the two populations was 1.5, with Malindi population mean of 2.1 and Tana River with 0.75. Inter population was 1.75 for both populations, 0.75 for Tana River and 2.26 for

Malindi with a co-efficient difference of 2.0 for both populations, Tana River 0.85 and Malindi 2.41.

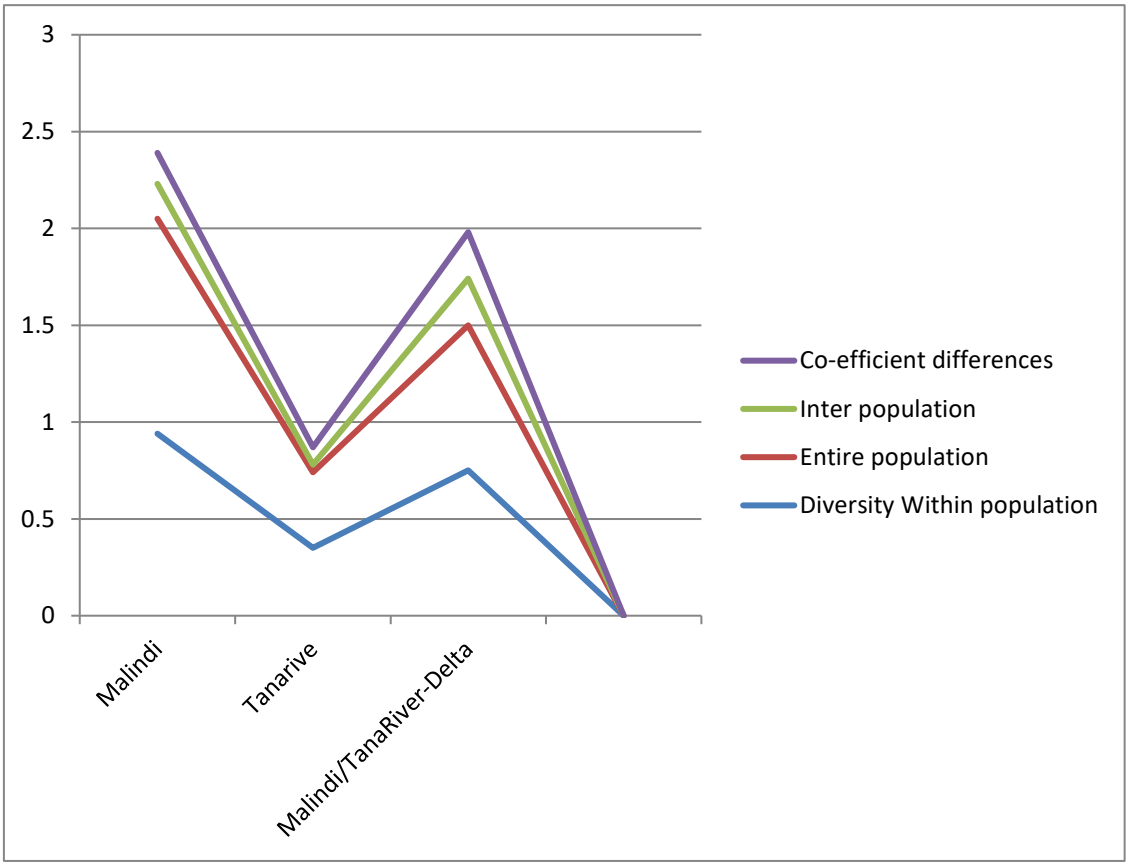


Figure 4.2: Evolutionary divergence of *W. bancrofti* 18S rRNA sequences of Mpirani-Malindi and Kipini-Tana River isolates

4. 4.4: Population mean and pair wise distances determination

Table 4.3 shows the distances between the sequences which were calculated using Maximum Composite Likelihood model (Tamura *et al*., 2004) as shown in Table 4.3 below.

Table 4. 3: Estimating evolutionary distance of Mpirani-Malindi and Kipini-Tana River Delta Isolates using Pair wise distance

ML1-2002												
ML2-2002	1.23											
ML3-2002	0.90	1.08										
ML4-2002	0.07	1.19	0.93									
ML5-2011	1.05	1.24	1.25	1.11								
ML6-2002	1.06	1.04	1.24	1.04	1.20							
ML9-2002	1.30	1.29	1.41	1.32	1.36	0.67						
ML11-2004	0.93	1.29	1.14	0.93	1.24	1.15	1.42					
TR1_2008	1.08	1.21	1.21	1.08	1.26	1.13	1.42	1.06				
TR2_2008	0.91	1.26	1.09	0.94	1.08	0.99	1.35	0.95	0.23			
TR3_2011	0.82	1.28	1.05	0.82	1.21	1.21	1.50	0.90	0.53	0.50		
TR4_2011	0.84	1.24	1.05	0.83	1.12	1.09	1.44	1.08	0.34	0.29	0.33	
TR5_2008	0.85	1.21	1.04	0.84	1.20	1.16	1.44	0.99	0.53	0.59	0.33	0.48
TR6_2011	0.84	1.38	1.02	0.82	1.25	1.07	1.38	0.93	0.47	0.44	0.21	0.28	0.30

Nucleotide sequences from 14 specimens were analysed. 1st, 2nd and 3rd codons and noncoding positions were included in the analysis. The final data set had 262 codons and noncoding positions.

Figure 4.3 shows the mean composition distance between the sample's population of 0.99 and within group mean distance was 1.11 for Malindi and 0.39 for Tana River. Between mean group distance was 1.1 and the net between group was 0 .35.

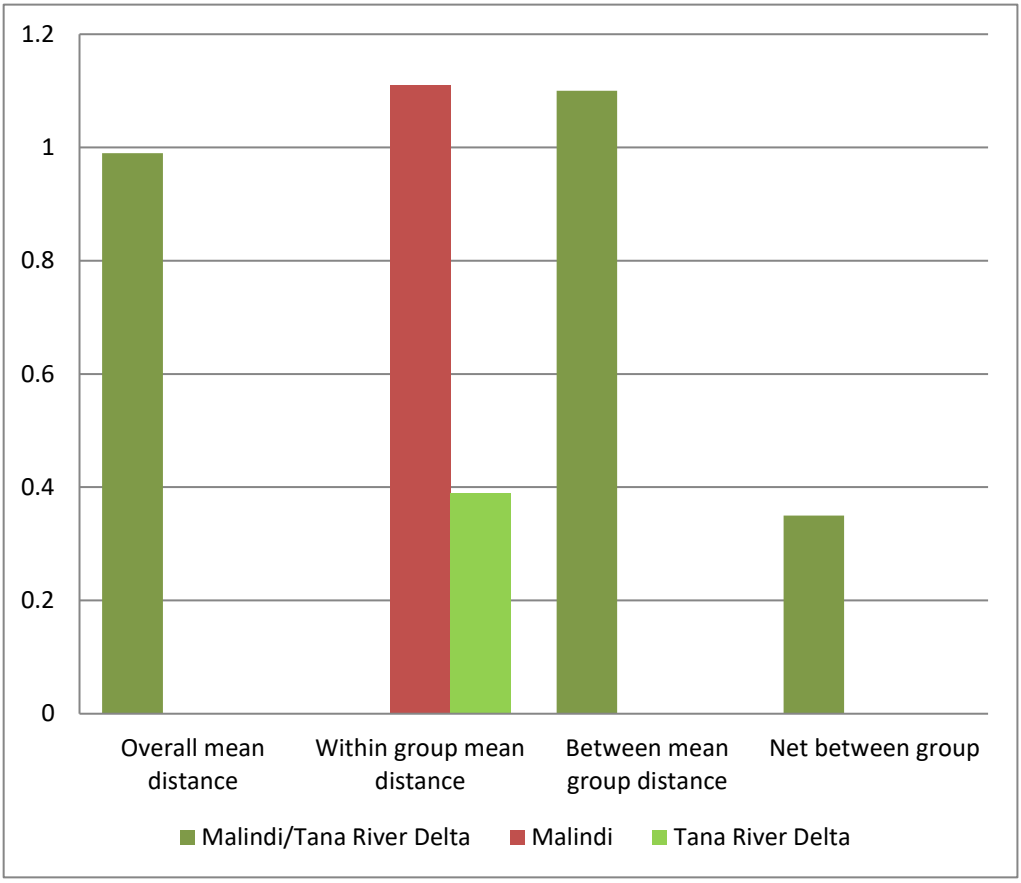


Figure 4.3: Mean distances of Mpirani-Malindi and Kipini-Tana River Delta isolate equences

Malindi isolates in red bar shows a greater distance compared to Tana River Delta isolates in light green bar. The darker green bar is the distance between Tana River Delta and Malindi Isolates

4.4.5: Molecular phylogenetic analysis of *W. bancrofti* 18S rRNA of Mpirani-Malindi and Kipini-Tana River Delta isolates

Phylogenetic tree was constructed using 14 *W. bancrofti* isolates form Malindi and Tana River Delta using MEGA 7 evolutionary analysis (Kumar *et al*., 2016). Maximum likelihood method by Jones, Taylar and Thornton (JTT) matrix-based model and a Bootstrap of 500 iterations were used to infer the evolutionary history of the sequences (Jones *et al*., 1992).

Figure 4.4 is a phylogenetic tree drawn to scale, branch lengths are measured in the number of substitutions per site. Association of clusters is given by the percentagies and the initial

trees for heuristic search were obtained by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using JTT model.

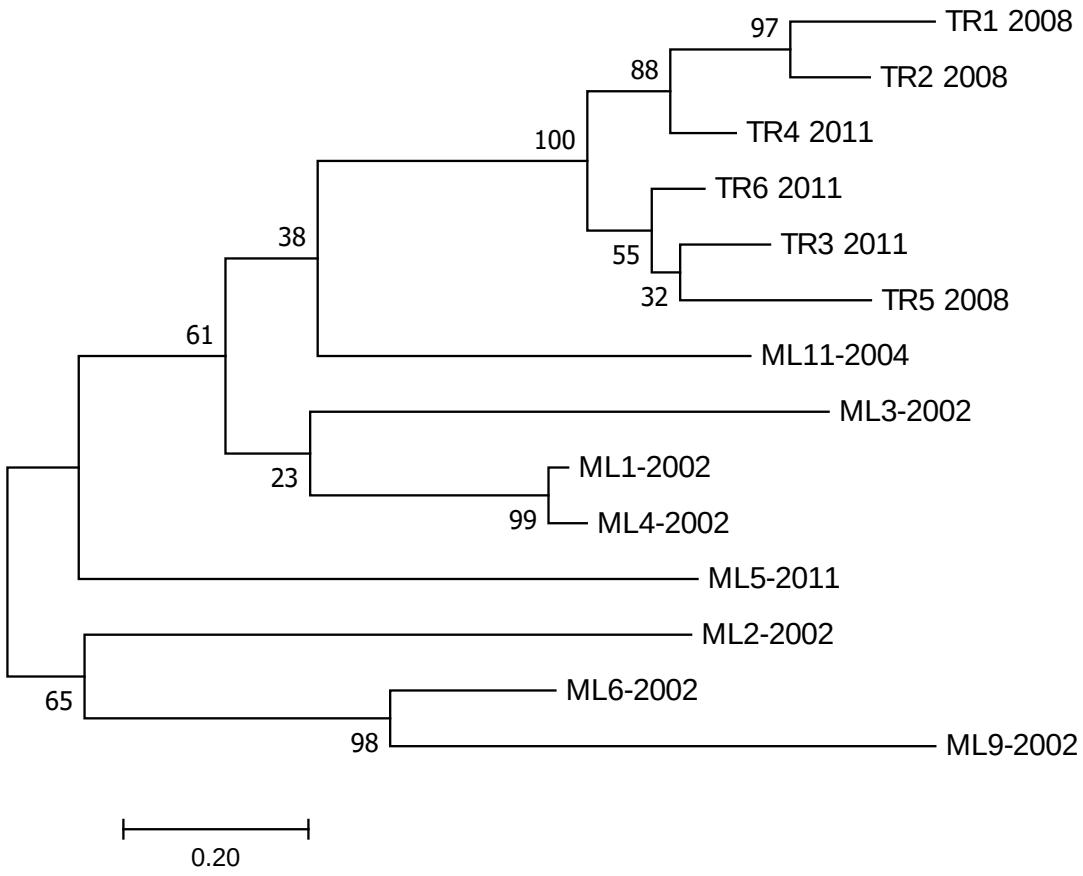


Figure 4.4: Phylogenetic tree of *W. bancrofti* isolates from Tana River Delta (TR) and Malindi (ML).

Phylogenetic tree based on 18S rRNA gene sequences was constructed using neighbour joining method by Kimura-2-distances in MEGA 7 programme. Numbers on the nodes are bootstrap values

4.4.6: Molecular phylogenetic analysis of Kenyan and related sequences from GenBank

Fourteen Kenyan isolates sequences and reference filarial sequences from GenBank (Accession Numbers; EU272178.1, M86642.1, DQ995498.1, DQ995497.1, M86643.1 and x16591.1) were aligned. Figure 4.5 shows a phylogenetic tree constracted to determine sequences relationship.

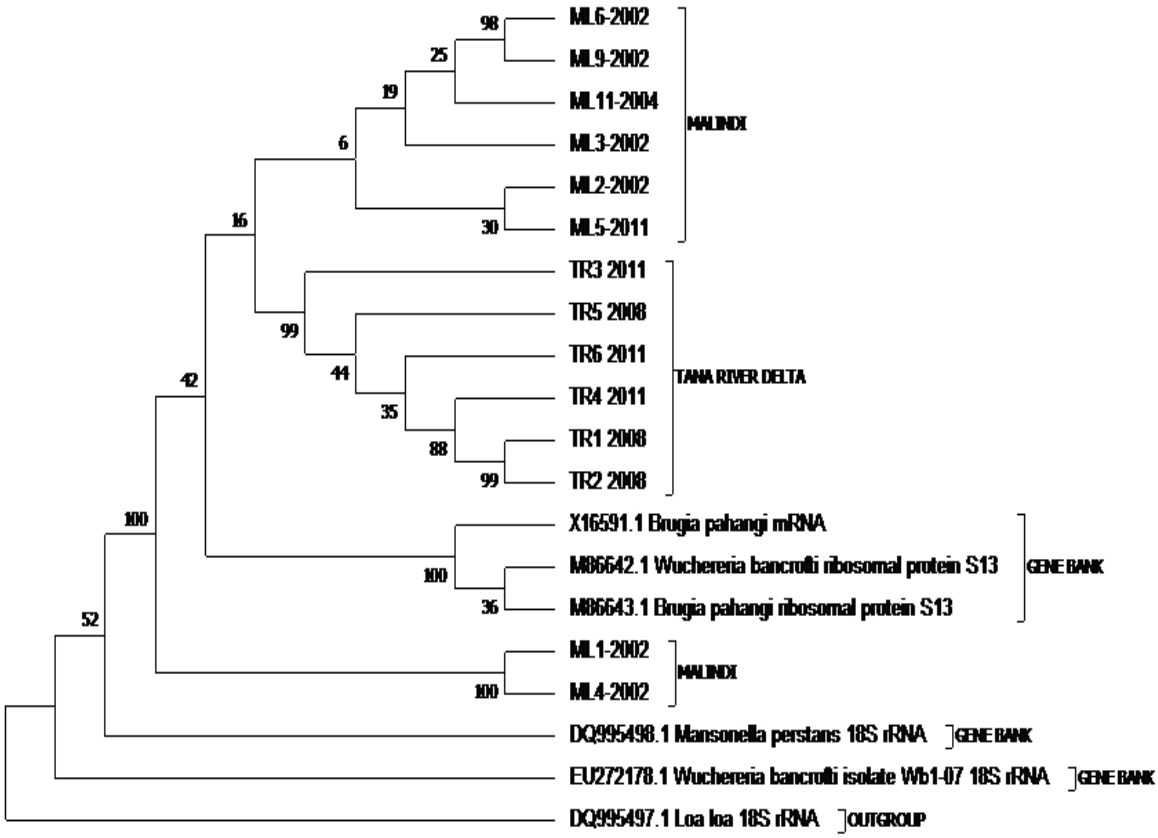


Figure 4.5: Phylogenetic tree of *W. bancrofti* 18S rRNA gene isolates from Mpirani and Kipini isolates

The phylogenetic tree was constructed using neighbour joining method by Kimura-2-distances in MEGA 7 programme. TR denotes Kipini-Tana River Delta isolates and ML-the Mpirani-Malindi isolates. Reference sequences from GenBank are indicated by accession numbers starting with letters DQ, EU, M & X. Numbers on the nodes are bootstrap values. Loa loa 18S rRNA sequence Accession Number DQ995497.1 was used as outgroup.

4.4.7 Selection and Neutrality test

Wuchereria bancrofti 18S rRNA sequence data was exposed to Tajima's (D) test statistics for neutrality selection analyses as described by Tajima (1989) and Nei (2000). Malindi and Tana River population sequences had a Tajima's (D) test of 4.149 and nucleotide diversity(π) 0.603 P< 1.0 at 269 segregating sites. Malindi populations had Tajima's D test

of 3.822 and nucleotide diversity(π) of 0.654 with P< 1. 0 and Tana River had Tajima's D test of 1.446 and diversity of 0.318 with p< 0.05.

4.5: Discussion

4.5.1; Genetic characterization of *W. bancrofti* 18S rRNA gene in isolates of Mpirani-Malindi and Kipini-Tana River Delta in Kenya

Human population infections and xenomonitoring are important aspects in filariasis control programmes. Infections monitoring allow identification of new transmission zones, assessing progress of the control programmes and determining when to stop control interventions when the elimination goal is achieved.

The Kenyan isolates from Malindi and Tana River Delta regions were assigned accession numbers on GenBank as shown in Table 4.1. These sequences form the first genetic data from 2 Kenyan endemic regions which will be used for identifying and comparing any genetic divergences and diversity in other studies. BLASTs results of the sequences found that the isolates identity range with those in GenBank was 79-98% (Table 4. 2). These results indicated that there was great genetic relationship between strains in 2 endemic regions of Kenya studied and other related parasites from other endemic regions as obtained from database. Genetic analysis of *W. bancrofti* isolates is essential as it provides insights of targeted regions for drugs, vaccines, diagnostic tools developments and for determining instances of drug resistance. These results of this study add information on the scanty data on *W. bancrofti* genetic variability of previous studies by Ramesh *et al.* (2012) and De- Souza *et al*. (2014). Parasite's structure was evaluated in the current study by comparing genetic distances within and between the two Kenyan populations. Overall mean composition distance between Malindi and Tana River isolates was 0.99 and that of within group mean distance was 1.11 and 0.39 for Malindi and Tana River Delta isolates respectively. It was fould that, Malindi isolates had a greater pairwise distance as compared to Tana River Isolates.These results indicate that, within Malindi group mean distance was

greater compared to the overall mean distance of the two Kenyan populations (Figure 4.3). A study by Rekha *et al*, (2006) showed that *W. bancrofti* population cluster in accordance to drug treatments. Malindi being a cosmopolitan region, human migration might have influence *W. bancrofti* structure by gene flow brought about by infected people movements or vector dispersal. Again, the differences in divergency may be attributed to the long period Malindi has been on MDA, from 2003-to date and Tana River from 2011 to date. Filariasis control programs uses a single dose of either combination of Albendazole and Diethylcarbamizine or Ivermectin, and sometimes the combinations of the three drugs. Heavy reliance on these drugs may result to emergence of resistance strains of the parasites due to drug pressure effects leading to genotypic and phenotypic changes in parasite population structure as ealier reported by Wolstenholme *et al*. (2004) and Kaminsky (2003). Thangadural *et al*. (2002) genetic variants of parasites with high genetic divergence and gene flow.

Human populations and vector migrations which have no borders are also factors which contributes to variations and development of drug resistance strains. Studies by Pichon (2002), reported that, MDA by DEC for over 50 years did not eliminate the disease in Polynesian Islands of Moorea and Maupiti due to development of resistant starins. Development of resistant strains to Ivermectin and Albendazole are more common in nematodes of veterinary importance (Schwab *et al.,* 2007), thus understanding genetic differences in strains is of much interests towards monitoring parasites responses to drugs. In their study, Schwab *et al.* (2007) compared resistant allels in *W. bancrofti* patients treated with a combined dose of Albendazole and Diethylcarbamizine with allels from untreated people in Ghana and Burkina Faso. The resistance allels were at 26.2% frequency in untreated populations and 60.2 % and 86.2% in patients treated with the combined drug once and twice respectively. They also observed that some untreated Ghanaian populations

had allel frequencies as low as 0.33%. Elimination programs by MDAs are adversely affected by the assumptions that parasites in a population are the same and can be treated by similar drug dose. Genetic heterogenecity within populations in similar geographical region requires appropriate chemotherapeutic strategies for filariasis elimination (Churcher *et al*; Hoti *et al*., 2008).

In this study, it was observed that isolates from Tana River clustered together an indication of little genetic variations (Figure 4.4) as compared to that of Malindi isolates which clustered into 5 different clusters. There were no differences with strains that were from similar geographical region as shown by isolates ML1 and ML4 which were collected from the same village. The isolates share high sequence identity with each other.

For the purpose of more phylogenetic comparison, reference sequences from the GenBank (NCBI) were retrieved and a phylogenetic tree constructed with the Kenya populations (Figure 4.5). Sequences ML1 and ML4 from Malindi populations in 2002 before the start of any MDA clustered closely with *Mansonella perstans* 18S rRNA, *Loa loa* 18S rRNA and reference *W. bancrofti* isolates (Accession numbers DQ995498.1, DQ995497.1 and EU272178.1) respectively (Figure 4.5). Closely related to isolates ML11 of 2004 after one round of MDA were parasites of *B. pahangi* ribosomal protein S13 with accession number M86643.1, *W. bancrofti* ribosomal protein S13 with acceccion number M86642.1 and *B. Pahangi* mRNA accession number X16591.1. From the constructed phylogenetic tree with related strains, Kenyan isolates from both Kipini in Tana River Delta and Mpirani in Malindi were found to have originated from *W. bancrofti* isolates W. b 1-07 18S rRNA accession number EU272178.1, from Brazil. This analysis used *Loa loa* 18S rRNA accession number DQ995497.1from Spain as the out-group. Evolutionary analysis showed that, there were close relation of the Kenya isolates with the related GenBank sequences retrieved as 18S rRNA is widely used to establish phylogenetic relationships.

4.6: Conclusion

This study provides genetic information on 18S rRNA fragment of *W. bancrofti* in Malindi and Tana River Delta endemic regions in Kenya. There is a high genetic relationship of the Kenyan isolates from Mpirani-Malindi and Kipini -Tana River Delta with sequences retrived from the GenBank. It is inferred from this study that 18S rRNA is highly conserved among the 14 isolates and thus a good target for diagnosis of *W. bancrofti* DNA.

4.7: Recommendations

More studies on genetic characterization of *W. bancrofti* actin genes are required from all other endemic regions in Kenya to track any genetic variations for diagnostic targets and drugs development.

CHAPTER FIVE: DESIGN AND OPTIMIZATION OF LOOP MEDIATED ISOTHERMAL AMPLIFICATION AND HELICASE DEPENDENT AMPLIFICATION ASSAYS AS POINT-OF-CARE TOOLS FOR DETECTION OF *WUCHERERIA BANCROFTI* DNA IN HUMAN BLOOD IN TANA RIVER DELTA, KENYA

5.1: Abstract

Introduction; Prompt and accurate diagnosis of parasitic diseases is paramount aspect for the success of any control intervetions. Lymphatic filariasis is controllable at early stages and there is no treatment for chronic stages such as lymphedemas, it is therefore, necessary to detect these parasites early and initiate mass drug administration for effective control. WHO recommends use of sensitive, accurate, simple and low-cost diagnostic tools for monitoring of control programmes. Isothermal amplification methods are being developed as point-of-care tools for diagnosis of lymphatic filariasis. Here, LAMP and HDA assays were developed and validated for field application.

Methodology: One hundrend and twenty-five blood samples from Tana River Delta were collected and used for optimization of the two isothermal assays. DNA extraction and amplification by cPCR, LAMP and HDA assays were performed. Assays sensitivity and specificity were calculated using 2x2 contigency tables and evaluated against cPCR as gold standard.

Results; LAMP assay had 92.3% sensitivity and 97.3% specificity with high kappa statistics agreement to cPCR. HDA assay had 78.6% sensitivity with 93.7% specificity and K_{value} of 0.67.

Recommendation; This study recomments use of LAMP method for point-of-care tool for *W. bancrofti* DNA detection.

5.2: Background information and literature review

Elephantiasis is stigimatizing disfiguring disease of a major psychosocial significance to both the victims and care givers. Detection of microfilariae in blood specimens collected from infected individual and mosquitoes is regarded as point-of-care gold standard method for active infections. The method is based on microscopic examination and morphological assessment of stained microfilariae from blood collected at night (2200pm-2000am) to coincide with nocturnal periodicity of microfilariae and mosquito feeding time. *Wuchereria bancrofti* specific circulating filarial antigens is detected using Immunochromatographic card tests or Alere Filariasis Test strip (FTS) (McCarthy *et al*., 2012; WHO, 2011).

Polymerase chain reaction assays are sensitive and specific methods though they are not used in clinical set ups due to their requirement of specialized equipments and well-trained personnel (Blaire *et al.,* 2019). Isothermal assays have been considered for point-of-care diagnosis because of their simple reaction schemes that require simple heating apparatus. LAMP assays are available for detection of *Loa loa*, *Onchocerca*, *Brugia* and *Wuchereria* species (Poole *et al.*, 2019; Drame *et al.*,2014; Takagi *et al*., 2011) though they are not widely used in resource limited areas characteristics of many endemic regions. Helicase Dependent Amplification (HDA), mimics *in vivo* DNA replication process. This method uses DNA helicase enzyme other than heat to denature double stranded DNA for primer hybridization and extension. This is a true isothermal method using constant temperature for amplification eliminating use of cycling temperature that requires thermo-cycler machine (Vincent *et al*., 2004). The process achieves amplification of many copies of DNA in a short period of time compared to PCR. There are several challenges faced with diagnostics of *W. bancrofti* despite of the availability of rapid and advanced tools. For instance, PCR assays are costly requiring expensive equipment, reagents that requires full

time refridgeration and the need for well-trained personnel. Most of the antibody and antigen test kits have lost sensitivity and are not readily available to many endemic countries. There is need for diagnostic methods that are simple, rapid, sensitive, and reliable for point-of care use.

This study therefore was conceived to optimize and evaluate LAMP and HDA as molecular isothermal amplification assays for diagnosis of *W. bancrofti* to aid in the disease transmission assessment in Kenya. The specificity, sensitivity and Kappa statistics of the two Isothermal methods were evaluated against conventional PCR as the 'Gold standard'.

5.3: Materials and methods

5.3.1: Study site

Blood samples were collected from Tana River Delta, a Kenyan endemic region which had high initial prevalence of 22.2% (Kagai *et al.,* 2008) before the beginning of MDA.

5.3.2: Ethical clearance

Acceptable ethical aspects were considered through out the study. Approvals were sought from Kenya Medical Research Institute Scientific and Ethical Review Unit, protocol number SSC. 2802.

5.3.3: LAMP primers

Primers used were designed by Takagi *et al*., (2011). The designed primers targeted 18S rRNA Species specific regions (Ssp1) on *W. bancrofti* complete sequence of accession number (AY297458) which yields 188-base pair product. Table 5.1 is the two sets of primers used in LAMP assay designing.

Table 5.1: LAMP Primer set targeting 18S rRNA of *W. bancrofti*

Primer	Sequence
FIP	CGACTGTCTAATCCATTCAGAGTG-TATCTGCCCATAGAAATAACTACG
BIP	TCTGTGCTGAATTTTTGTGGATTG-CCAAACTAATTGTAAGCAGTCTT
F3	TTTGATCATCTGGGAACGT
B3	AAGCACCTTAAATCTGTCAAT

5.3.4: LAMP primer specificity testing

Specificity of LAMP outer primers (F3 &B3) were tested by cPCR. The primers were specific for *W. bancrofti* detection as indicated by positive and negative controls. Similarly, the tested specimens also gave results as expected; positive for positive and negative for negative specimens as they were earlier tested by primers designed for PCR as NV1 and NV2 as in Figure 4.1 of Chapter 4.

5.3.5: Blood samples processing and amplification

Blood samples collected from Tana River were used to optimize and validate both HDA and LAMP assays. Alcohol precipitation method was used for DNA extraction from blood specimen as described by Datta *et al*., (2007). Concentration of extracted DNA was determined as described in chapter 4 section 4.3.6. *Wuchereria bancrofti* DNA was amplified by LAMP and PCR assays for comparison and detected by both agarose gel electrophoresis and SYBR 1 Green dye.

5.3.6: *Wuchereria bancrofti* DNA amplification by convetional Polymerase chain Reaction

The reactions were performed as originally reported by (Zhong *et al*., 1996) using NV-1 and NV-2 primers as detailed in chapter 3 section 3.4.4.

5.3.7: LAMP optimization for amplification of *W. bancrofti* 18SrRNA

Loop mediated isothermal method was optimized for field validations by varing reaction temperatures from 60^{o}C to 65^{o}C and time between 30 to 60 minutes. Different primer concentrations of 5 to 10 pmol/µl for F3 and B3 and 20 to 40 pmol/µl FIP and BIP primers were tested. Reaction mix of final volume of 25µl was prepared, the master mix contained primers 1µl of FIP and BIP at 40 pmol/µl each and 1 µl of F3 and B3 at 5 pmol/µl each,

0.5µl of Bst I large fragment DNA polymerase, 1µl of 1mM dNTPs and 17.5µl of 1× reaction buffer. Two microliters of the template DNA were added to the reaction mix then incubated at 60^{o}C for 60 minutes on a heat plate. During optimization process 7.5 % DMSO was added as described in an assay by De-Guo *et al*. (2015) to reduce false positives as a result of primer dimers.

5.3.8: Use of closed tube lyophilized LAMP reaction buffers

A more suitable LAMP assay for field application is by use of a commercial isothermal amplification kit with lyophilised reagents (Eiken chemical Co., Tochigi, Japan). The reaction tube contains all reagents except the primers and the template. This reduces handling of many reagents which may result to contaminations. SYBR 1 green dye (Invitrogen, Carlsbad, CA) was added into the tube which is used for visualization of amplicons. SYBR green dye changed from blue to green colour in the presence of DNA and no colour change in absence of the DNA.

5.3.9: LAMP specificity testing

To test for the assay specificity, amplifications by LAMP were run using *W. bancrofti* specific primers on DNA of other relate parasites as described in chapter 3.

5.3.10: LAMP sensitivity testing

LAMP assay sensitivity was determined by establishing the lowest detectable limit. Ten-fold serial dilutions were prepared and amplifications by LAMP and PCR performed. Clinical specimens from 125 participants were tested for assay accurancy evaluation as in section 3.5.1 of chapter 3.

5. 4: Results

5.4.1: LAMP amplicons detection

Detection was done by visualization using florescence dye, SYBR 1 Green and by gel electrophoresis. Figure 5.1 shows the florescence colour change during detection while Figure 5.2 shows the LAMP products on agarose gel electrophoresis, with positive results showing a ladder like stream of bands on the gel and without notable bands for negative specimens.

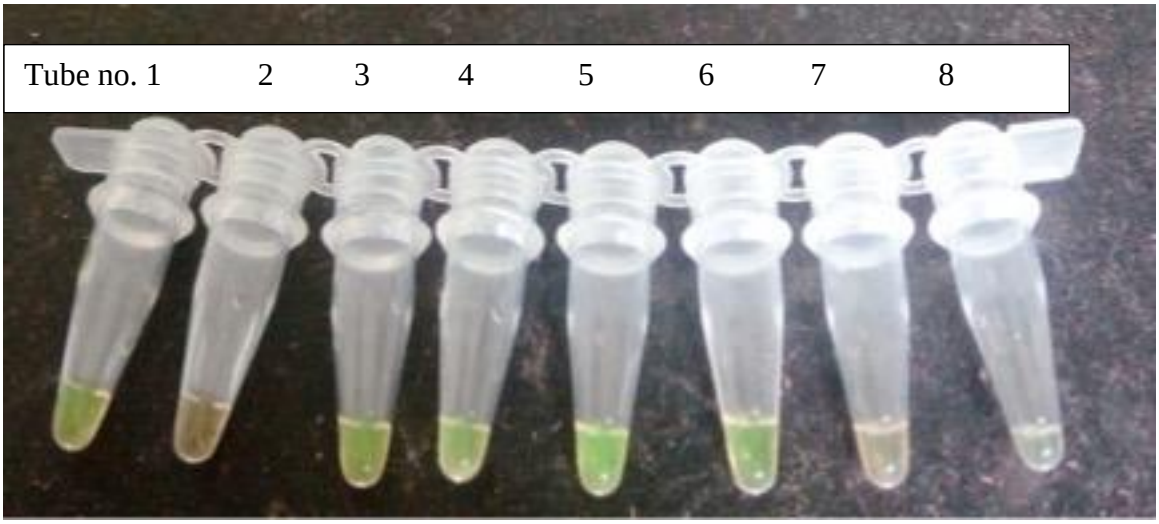


Figure 5.1: Florescence dye detection (Open Tubes) of amplified 18S rRNA *W. bancrofti* DNA fragment by LAMP assay

By use of florescence dye, the colour changed from orange to green in presence of *W. bancrofti* DNA and it remained orange in the absence of an amplicon. Tube No. 1 is *W. bancrofti* positive DNA control, tube 2 is negative control, tubes 3,4,5,6 are positive specimens (with *W. bancrofti* DNA) and tubes 7 & 8 are negative specimens

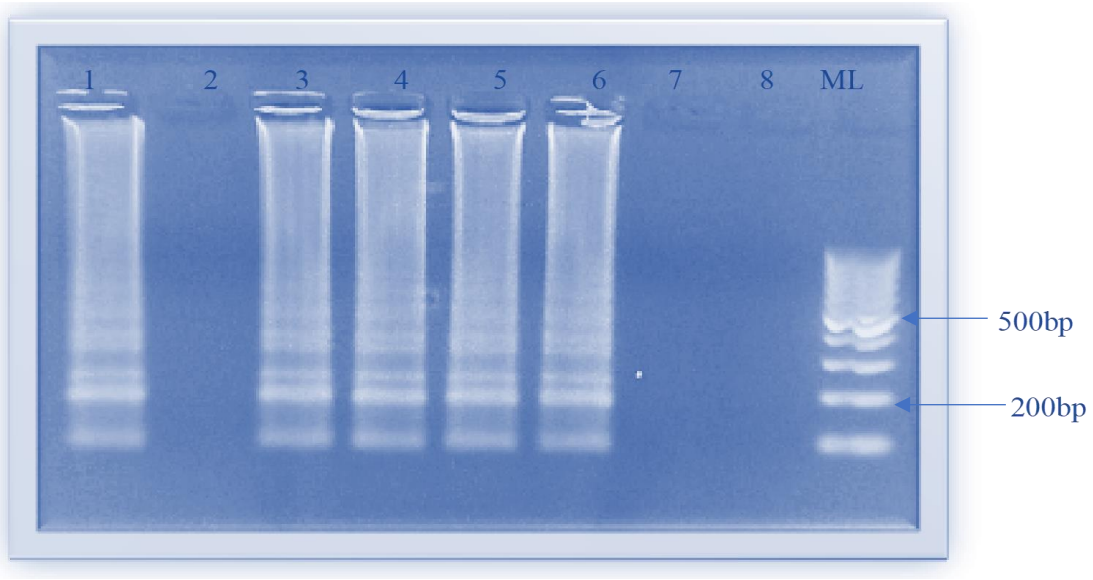


Figure 5.2: Detection of *W. bancrofti* 18S rRNA fragment amplified by LAMP assay on agarose gel electrophoresis

Lane 1 represents *W. bancrofti* positive DNA control, lane 2 represents Negative control, lanes 3, 4, 5 & 6 represents *W. bancrofti* amplified DNA from positive specimens and lanes 7 & 8 have no amplifications thus negative specimens. Lane ML was DNA standard marker (100bp ladder).

Closed tubes with lyophilized reagents were used in addition to species specific primers for amplification of *W. bancrofti* 18S rRNA in a DNA (Template), and the SYBR green dye. Figure 5.3 shows LAMP reaction before incubation and Figure 5.4 shows reaction after incubation at 60^{o}C for 60 minutes.

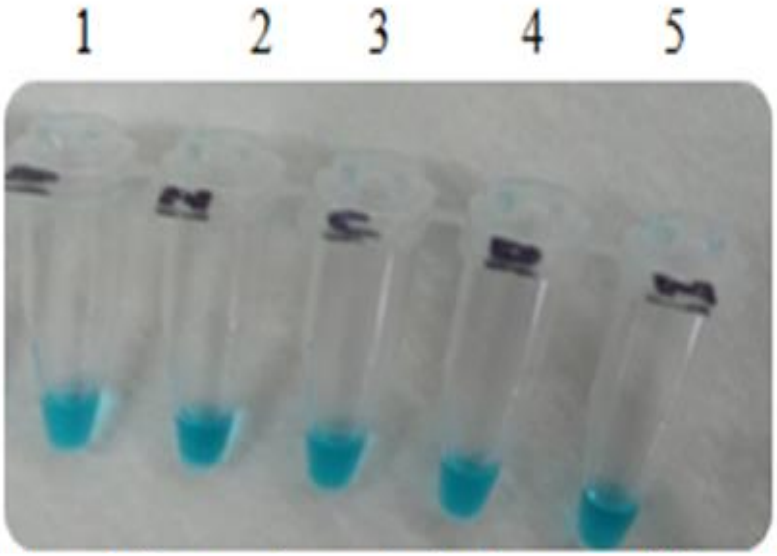


Figure 5.3: LAMP reaction mixture in lyophilized tubes (Closed tubes) before incubation. The reaction mixes in tubes 1 to 5 are all blue in colour.

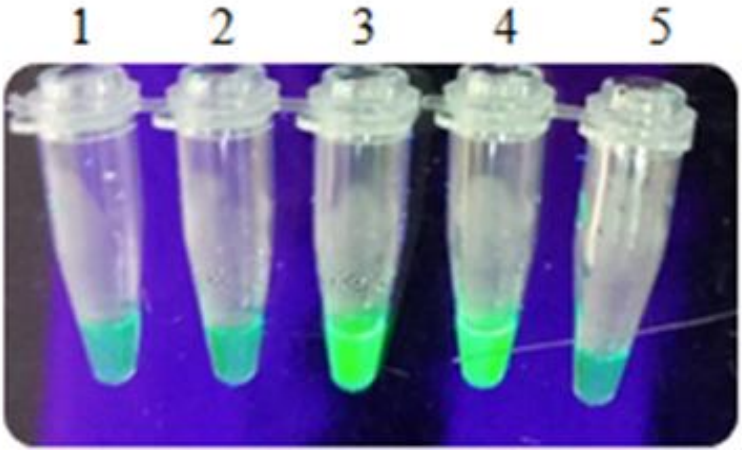


Figure 5.4: LAMP reaction mixture after incubation at 63^0C for 60 minutes,

Tubes 1 and 2 are negative specimen, Tube 3 is *W. bancrofti* positive specimens, 4 is *W. bancrofti* positive DNA control and 5 is a negative control. The colour changes from blue to green in presence of DNA amplicons.

5.4.2 LAMP Assay evaluation for amplification of *W. bancrofti* 18S rRNA fragment

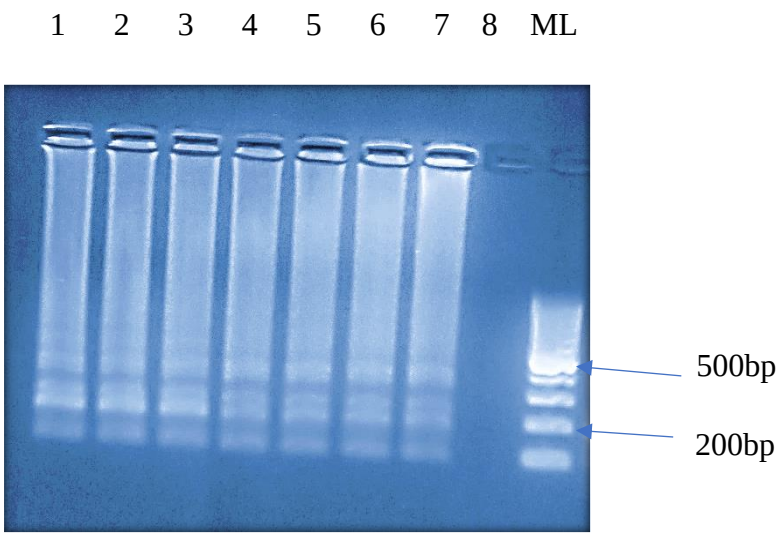


Figure 5.5: LAMP assay sensitivity testing of *W. bancrofti* from Tana River Delta isolates

Lane; 1= $1/10^0$, 2=$1/10^1$, 3=$1/10^2$, 4=$1/10^3$, 5=$1/10^4$, 6=$1/10^5$, 7=$1/10^6$, 8 =$1/10^7$, ML is Molecular ladder (100bp). The assay detected DNA copies from 1 ($1/10^0$) up to 1/1000000 ($1/10^6$) copies

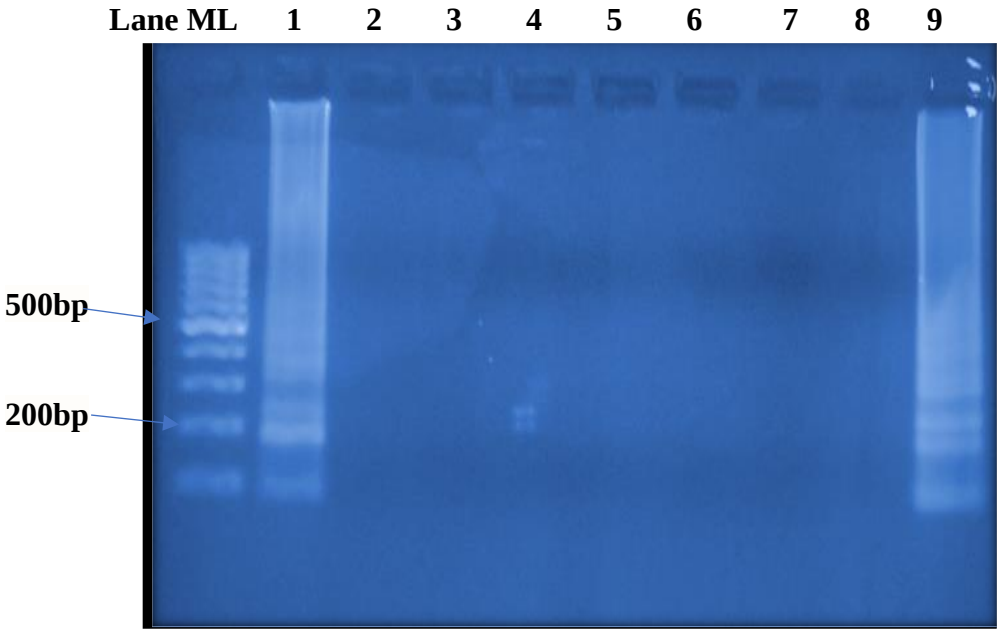


Figure 5. 6: Specificity of *W. bancrofti* LAMP assay testing from Tana River Isolates

Lane ML represents DNA size standard molecular weight ladder of 100bp, lane 1 shows amplification of *Wuchereria bancrofti* and Lanes 2-7 represented non-*Wuchereria bancrofti* species which did not show any amplification. Lanes 2- *Echnococcus granulosus*, 3-*Brugia malayi*, 4- *Anopheles gambiae*, 5- *Schistosoma mansoni*, 6-*Plasmodium falciparum* and 7-*Trichuris trachura* lane 8 represents negative control and 9 represents positive *W. bancrofti* DNA control.

Duration taken by LAMP assay to amplify DNA was evaluated, the results obtained within

30 to 60 minutes of incubation were relatively the same as shown in Table 5.2 below.

Table 5.2: LAMP assay Time optimization in minutes

Sample	Dye Colour change on incubation/duration of time		
	Incubation for 30 minutes at 60^{o}C	Incubation for 40 minutes at 60^{o}C	Incubation for 60 minutes at 60^{o}C
W. bancrofti positive DNA control	Green in colour	Green	Green
Positive specimen (K19)	Light Green	Green	Green
S. Mansoni	Orange	Orange	Orange
Negative control	Orange	Orange	Orange
Master mix –Blank	Orange	Orange	Orange

An indication of colour change from orange to green was observed within the first 30 minutes of incubation at 60^{o}C and there was no much change in colour even with further of incubation.

5.4.3: Clinical analysis of LAMP assays in the diagnosis of *W. bancrofti* DNA in samples collected from Tana River Delta

To clinically analyse LAMP assays, 125 patients' specimens were analysed by LAMP and PCR reaction as the 'gold standard'. Out of the specimens tested, 13 specimens were positive by PCR while 15 were positive by LAMP. A total of 112 samples were negative by PCR whereas 110 specimens were negative by LAMP assay. From these tests, clinical LAMP sensitivity 92.3% with a specificity of 97.3% at 95% confidence interval and a power of 1. LAMP had a Cohen's Kappa co-efficient of 0.84 shown in table 5.3.

Table 5.3: 2x2 Contingency table comparing LAMP technique and conventional PCR for diagnosis of *W. bancrofti* DNA

LAMP results	PCR the 'Gold standard' results		
	Positive (+ve)	Negative (-ve)	Totals
Positive (+ve)	12	3	15
Negative (-ve)	1	109	110
Totals	13	112	125

Sensitivity; 12/13= 92.3% and specificity; 109/112= 97.3%
Kappa statistics value is 0.84

5.5: Evaluation of Helicase Dependent Amplification (HDA) Assays for point-of-care detection of *Wuchereria bancrofti* DNA in human blood in Kenya

5.5.1 HDA design and flow

Helicase dependent amplification is an isothermal method that can amplify DNA at a constant temperature using only two primers. It depends on Helicase enzyme to unwide the double stranded DNA to single strands making it a true isothermal method without the need for heat denaturation of the double strands. Other isothermal methods such as LAMP and SDA are known to use 4 to 6 primers for amplification making the cycles abit complected compared to HDA cycle (Notomi *et al.,* 2000; Walker *et al.*,1992). In this assay, hybridization probes are used to confirm true positives in amplifications by allowing inclusion of competitive internal controls. HDA allows for the inclusion of competitive internal controls (CIC) in its assays. The internal controls help in determining a true

negative results and invalid results, which improves the test specificity. Enzyme Bst DNA polymerase used in HDA reactions is more tolerant to inhibitors as compared to Taq polymerase used in PCR assays.

In the reaction, the double strands are separated by DNA helicase enzyme and are coated by single stranded DNA binding proteins (ssDNA). In this system, strands of duplex DNA are separated by a DNA helicase and coated by single-stranded DNA (ssDNA)-binding proteins. Forward and reverse primers hybridise to each end of the target region on the template which are then extended by DNA polymerase using nucliotides to synthesize new strands. Figure 5.7 shows the process of HDA amplification by helicase enzyme separating the double helix for primer binding.

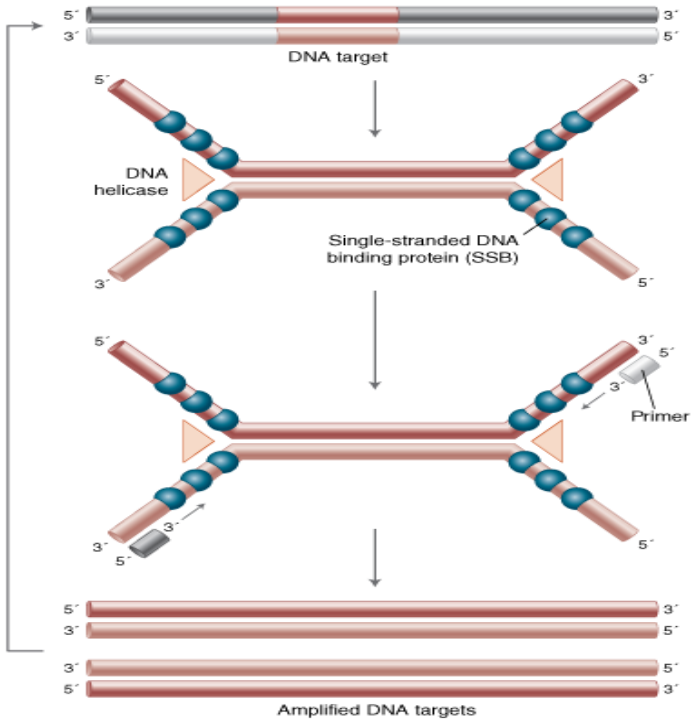


Figure 5.7: Diagrammatic illustration of HDA systematic replication

Source; *https://www.google.com/imgres*

5.5.2: HDA Primer designing

Well designed primers are needed for successful HDA amplifications. In this study, primers for HDA reactions were designed using Explorer V3 to target 18S rRNA gene in

W. bancrofti sequence. Five sets of paired primers per set were designed following primer designing instructions for HDA assays. Five pairs were tested and one set was selected for running the reactions. The following primer sets were used;

Forward primer; 5' -TATTGATAGTGTACGGGAGGGGTTTGG-3'

Reverse primer; 5' -GCTTCAGGCTTCTCTCATTTCACCACT-3'

5.5.3: Time considerations

Amplification process highly depends on the primers other than duration of the reaction. A number of factors have been found to increase the speed of reaction including additives such as Ficoll and the purity of the template making the reaction as short as 20 minutes or less.

5.5.4: HDA reactions for amplification of *W. bancrofti* DNA

DNA extraction was done as described in chapter 3 section 3.4.6. The reaction tubes containing reaction mix were incubated at 60^{o}C for 2 hours. Figure 5.8 shows size fragmentation of the amplicons on 2% agarose gel.

5.6: HDA Results

5.6.1 Results of HDA amplification assay, analytical sensitivity and specificity for detection of *W. bancrofti* DNA

Results of the HDA amplification assay are shown in figure 5.8 while Figure 5.9 shows the bands detected following 10-fold serial dilutions of the DNA. Figure 5.10 shows specificity testing of HDA assay reaction with only specimen containing *W. bancrofti* DNA amplified.

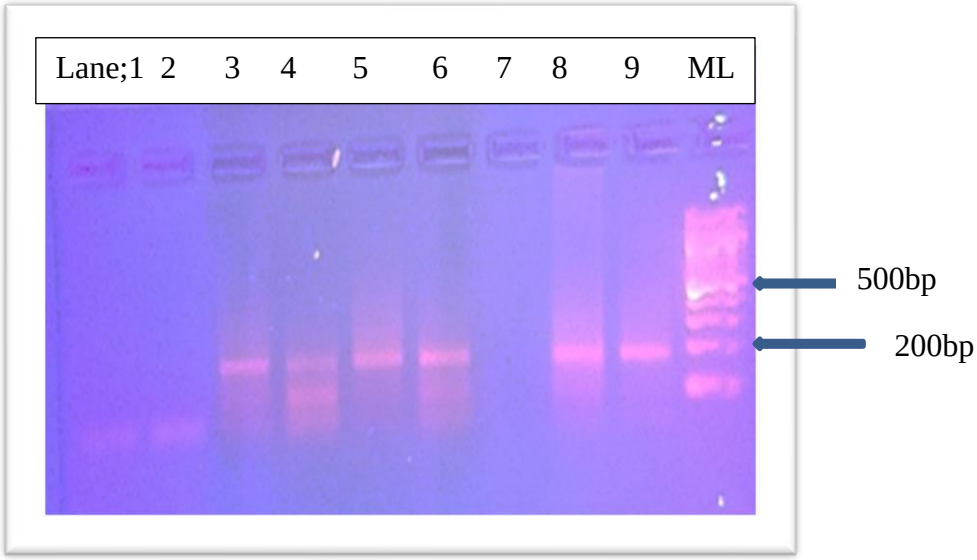


Figure 5.8: *W. bancrofti* amplicons by HDA assays detected on a 2.0% agarose gel electrophoresis

Lane 1 represents the negative control, 2 & 7 are negative specimens, 3,4,5,6 & 8 are positive specimen containing *W. bancrofti* DNA, Lane 9 is *W. bancrofti* positive DNA control & ML is DNA Molecular size marker (100bp ladder).

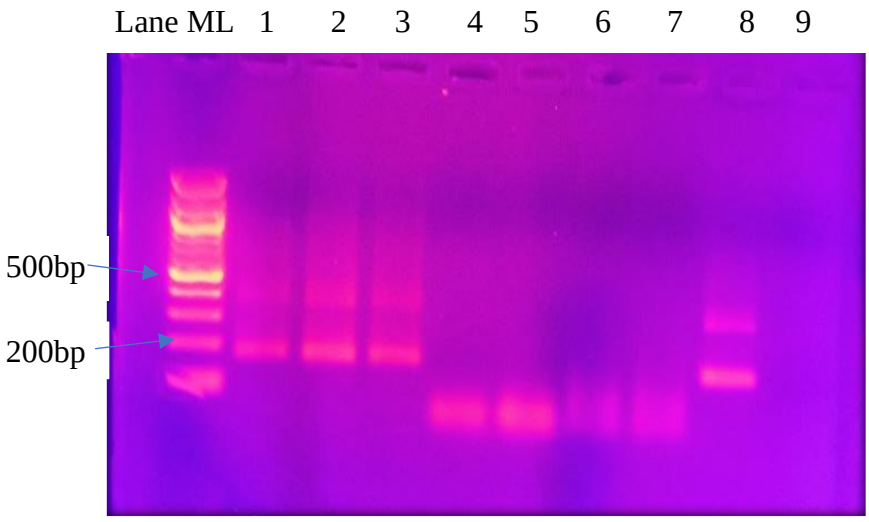


Figure 5.9: Agarose gel electrophoresis for analytical HDA sensitivity testing of *W. bancrofti* DNA

Lanes; ML is DNA molecular size standard (100bp ladder), 1= 10^{-1}, 2=10^{-2},3=10^{-3}, 4=10^{-4}, 5=10^{-5}, 6=10^{-6}, 7=10^{-7}, 8 = *W. bancrofti* positive control and lane 9 represents a negative control. The assay was able to detect up to 1/1000(1/10^{3}) copies of *W. bancrofti* DNA. With no amplification at DNA diluent 10^{-4} to 10^{-7} lane 4 to lane 7 while lane 8 represented *W. bancrofti* DNA control and lane 9 was a negative control.

5.6. 2: Clinical sensitivity and specificity testing of *W. bancrofti* DNA

Statistical sensitivity and specificity of HDA was tested and computed using 125 patients' blood from Tana River County as recorded in Table 5.4.

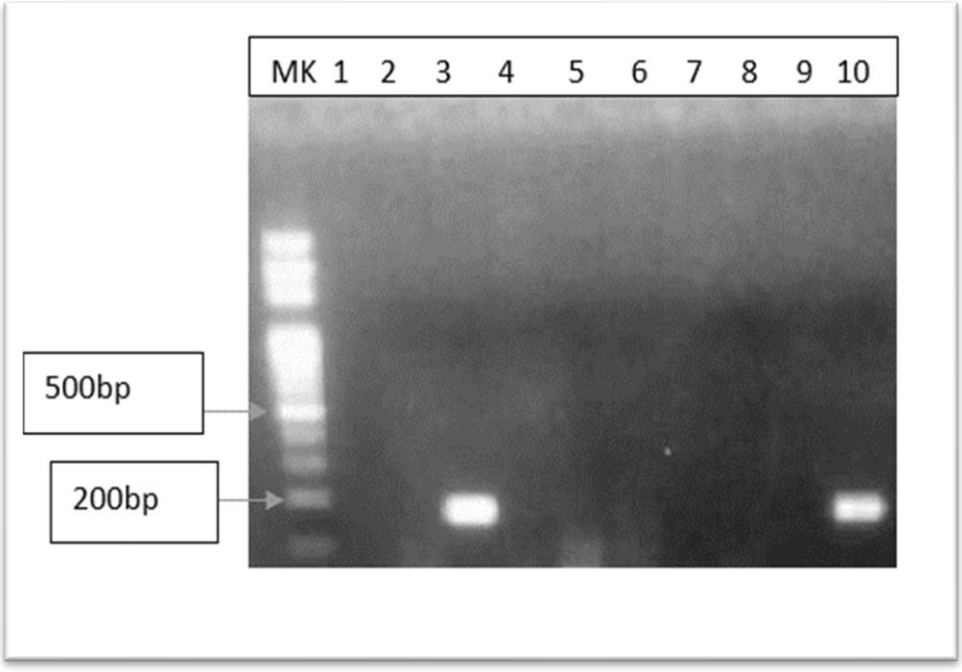


Figure 5.10: Agarose gel electrophoreses for analytical HDA specificity testing of *W. bancrofti* DNA

Lanes; MK – DNA molecular size standard marker (100bp), 1-*Brugia Malayi*, 2-*Schistosoma mansoni* 3- *Wuchereria bancrofti* DNA, 4 -*Plasmodium falciparum*, 5-*Trichuris trachura*, 6- *Anopheles gambiae*, 7- *Leishmania donovani*, 8- *Echinococcus granulosus*, 9- Negative control,10 – *W. bancrofti* positive control.

Table 5.4: 2x2 Contingency table comparing HDA technique and cPCR

HDA TEST	PCR TEST		Totals
	Positive	Negative	
Positive	11 a	7 b	a+b = 18
Negative	3 c	104 d	c+d =107
Totals	a+c=14	b+d=111	a+b+c+d= 125

Clinical sensitivity was given by= a/a+c 11/14= 78.6%
Specificity was given by d/b+d 104/111= 93.7%
Kappa statistics value is 0.67

5.7: Discussion

Loop-mediated isothermal amplification and helicase depended amplification assays were optimized in this study. LAMP assay allows DNA amplification at a constant temperature by use of Bst DNA polymerase enzyme while HDA uses helicase enzyme separate the double helix DNA before amplification takes place. With LAMP reactions, visual observations of turbidity or colour change in the reaction results are possible eliminating post amplification procedures. SYBR 1 Green dye and gel electrophoresis detection results were as shown in Figures 5.1 with its gel electrophoresis representation in Figure 5.2. In presence of DNA, the pH of the reaction mix changes resulting to colour change of the intercalating dyes. When florescence dye is use, the colour changes from orange to green while with SYBR green dye changes form blue to green in presence of amplicons. These results were in accordance with the reports by Notomi and Colleagues (2000). Ethidium bromide used in gel visualization is carcinogenic, minimizing it's use is an important aspect for public health safety. Figure 5.3 shows reaction mix in closed tubes with lyophilized reagents which were blue in colour before incubation, after incubation at 63^{o}C for 60 minutes, the colour changed from blue to green in presence of *W. bancrofti* DNA and remained blue in absence of *W. bancrofti* as viewed under ultraviolet light (260nm) (Figure 5.4). LAMP reaction in lyophilized reagents has added advantage in that there is minimal handling of reagents hence less chances of contaminations. The results of this study were similar to studies on LAMP colorimetric test by Poole *et al*., (2017) and Goto *et al*., (2009). LAMP reactions could amplify DNA within 30 minutes as shown in Table 5.2, an indication that the reaction can be performed in shorter time than what is indicated by in ealier studies by Notomi *et al*., (2000). The sensitivity of LAMP and HDA was tested by 10-fold serial dilution of the template with an initial concentration of 50.0ng/µl which is equivalent to 200pg, approximately 1(one) DNA copy. The developed LAMP assay had a

sensitivity of 92.3% at 95% confidence interval and at power of 1 (Table 5.3). The developed LAMP assay had a specificity of 97.3% as amplification was only in specimens containing *W. bancrofti* DNA as shown in table Table 5.3. LAMP reactions in this study had a Cohen's Kappa statistics value of 0.84 which is a strong agreement between LAMP and PCR which was used as the 'gold standard method'. Helicase dependent amplification showed a sensitivity of 78.6% with a specificity of 93.7%. HDA had Cohen's Kappa statistics value of 0.67, showing some good agreement. Comparing LAMP and HDA to PCR as the gold standard, LAMP had a higher sensitivity and specificity and also a strong agreement with PCR (K_{Value} 0.84) as compared to that of HDA which had a Lower K_{value} of 0.67. This suggests that LAMP assays can be used to substitute PCR in resource limiting regions.

LAMP has some advantages over PCR as it can be performed using simple apparatus such as heat block or a simple water bath at a constant temperature. LAMP amplicons can be visualized using pH sensitive dyes or turbidity of the reaction mixture, thus, LAMP method has proved ideal for use in a field set up without the need of agarose gel systems. Most of PCR methods have not been used in a field set up because of the requirement of expensive thermo-cycler and the need for trained personnel. Use of lyophilized close tube makes LAMP even more suitable for field operations as the reagents are more stable even with high temperatures, no refridgeration is required. LAMP assay described here for detection of *W. bancrofti* has shown a greater potential for use in the poorly equipped laboratories. The lowest detectable limit by LAMP was 10^{-6} equivalent to 1 microfilaria per 200µl (Figure 5.8) and by HDA was 10^{-3} (Figure 5.9). High rate of formation of non-specific amplicons was the major challenges during the assays optimization as cross contaminations were high due to exponetial amplifications. Challenges highlighted in this study were similar to those found by Nyan *et al*. (2014) and Angamuthu *et al*. (2011). To improve

LAMP specificity, 7.5% DMSO was added to the reaction mix to reduce the false positives. The use of closed tubes and intercalating dye for visualizations eliminates the need for electrophoresis and this also reduces contamination. With LAMP, it was also found that a touch down temperature of 94^0C was needed to denature the template before adding the enzyme at a lower temperature 4^oC.

HDA was a true isothermal DNA amplification method as DNA helicase could separate double-stranded target DNA without the need for raising the temperature. The template–primer inter-dependent nature of HDA results to non-specific amplifications as compared to PCR resulting to a lot of false positives if primers are not well designed. Use of DMSO, betaine or sorbitol reduce DNA secondary structures facilitating primer annealing (Green and Sambrook, 2019).

5.8: Conclusion

LAMP and HDA assays for amplification of *W. bancrofti* 18S rRNA fragment in human blood were optimized. LAMP assays had high sensitivity and specific for only *W. bancrofti*. Lyophilised reagents in closed tubes were very promising with reduced cross contaminations and they do not require cold chain as compared to reagents for cPCR. HDA had a lower sensitivity and specificity when compared to that of LAMP assays with cPCR as the gold standard. LAMP method if employed can be of much help to GPELF in achieving its goals of elimination. LAMP assay was therefore, selected over the HDA assay for further field validation.

5.9: Recommendations

This study recommends use of lyophilized premix to avoid cross contamination which was a major challenge through our study during the open tube analysis. In LAMP assays optimization, addition of DMSO 7.5% is useful in minimizing false positives.

CHAPTER SIX: FIELD VALIDATIONS OF LAMP ASSAYS AS POINT-OF-CARE TOOL FOR *WUCHERERIA BANCROFTI* DIAGNOSIS IN HUMAN BLOOD AND MOSQUITOES IN MATAYOS AND TANA RIVER DELTA IN KENYA

6.1: Abstract

Introduction; The main objective here was to validate LAMP assay for point-of-care diagnosis of *W. bancrofti* in a field set up.

Methodology; Field collected specimens from Busia and Tana River Delta were tested by LAMP assay. The assay was compared with PCR and gel electrophores.

Results and Conclusions; Clinical LAMP assay sensitivity was 91% and specificity was 99%. LAMP was found to have a detection limit of 10^{-6} in human blood and 10^{-4} in mosquitoes. LAMP was found to be a better method for point-of-care use that can substitute PCR assays.

Recommendation; This study recommends the use of LAMP in clinical set up in resource limited endemic regions.

6.2: Introduction

Diagnosis and treatment of filariasis depends on the geographical setting and availability of cost-effective medical facilities (Tang *et al.,* 2018). Experienced health care providers in an endemic setting are able to diagnose filariasis based on clinical presentations, however point-of-care tools are important for confirming infections (Belard *et al.,* 2016). To achieve elephantiasis elimination goals, effective monitoring and evaluation of MDA programs is key to reduce instances of infections resurgence. Morphological identification

of microfilariae and immunological antigen detection are usually cumbersome, time consuming and can lead to misdiagnosis.

To assess the progress of filariasis elimination program, infection rates in human and vectors is monitored (Weil and Ramzy, 2007; Weil *et al*., 2008). Mosquitoes harbor microfilariae of stage 1 to stage 3 (L_1-L_3) that are transmitted to human hosts when taking blood meal. Xenomonitoring has been used in monitoring progress of elimination programs (Chambers *et al*., 2009; Bockarie *et al*., 2007). Dissection of mosquitoes in search of larvae is not sensitive enough for detecting infection and infectivity rates in low transmissions, it is also tedious when dealing with large numbers of mosquitoes. Xenomonitoring by PCR assays to detect *W. bancrofti* DNA is an indirect measure of filarial infection rates in human populations and detection of L_3 stages gives the measure of transmission (Ramaiah and Edwin, 2013).

In this study, LAMP assay was validated for field application for *W. bancrofti* diagnosis in human blood samples and in mosquitoes.

6.3: Materials and methods

6.3.1: Study site

Patients blood samples and vector mosquitoes for this objective were collected from Busia and Tana River Counties. Details of the study sites are found in section 3. 1 of chapter 3.

6.3.2: Study approval and ethical consideration

The study was approved by KEMRI- Scientific and Ethical Review Unit, protocol number SSC. 2802 for Tana River Delta and number SERU 3561 for Busia studies. Willing participants gave a written consent after being explained on the purpose of the study, assent forms for children were signed by the parents or their guardian.

6.3.3: Study design

A purposive, case and cross-sectional based study that aimed at determining prevalence of lymphatic filariasis in Matayos constituency, Busia County and Tana River Delta in Tana River County.

6.3.4: Survey strategy

Participants' house to house approach was used during recruitment and samples collection. This was done with the help of village elders and local community volunteers of each clinical unit who were assisting in mobilizing the villagers and identifying patients with clinical signs. The baseline for participant's selection was the chronic cases and the people living closely to the affected individuals. A total of 11 Community units/villages in Matayos consitutiency and 5 villages in Tana River Delta were involved in the study. The figure below shows field activities taking place during blood collection. In figure 6.1 (a), Community health volunteers are being educated on lymphatic filariasis transmission, signs and control while figure 6.2 (b) is blood collection exercise.

(a) (b)

Figure 6.1: Community mobilization and blood sampling exercise in Matayos and Tana River Delta (a) In community mobilization by educating CHV in Busia- Matayos sub-county and (b) Clinician bleeding a patient in Tana River Delta, Wema- Municipality. **Photo credit**; Nancy Kinyatta.

6.3.5: Mosquito sampling

Indoors and outdoors mosquitoes were collected within the compounds of the study participants who turned out to be positive for circulating filarial antigens. Consent was obtained from the household heads before setting out the traps. CDC light traps were set from 6pm and collected at 6am. For the in-door collection, the traps were set inside the patients houses near bed sites to maximize the chances of getting fed infected mosquitoes. For the out-door collection, the traps were set on doorsteps and also near the cow sheds. On removing the traps, they were sprayed with chloroform for killing the mosquitoes before they were identified.

The nature of the houses had a great influence on number of mosquitoes collected indoors. Figure 6.2 represents houses in study area where indoor mosquito collections were done.

(a) (b)

Figure 6.2: Images of some of the participants' houses where blood sampling and mosquitos' collections were done. Image scredit; Nancy Kinyatta
A grass thatched mud build house and (b) Corrugated Iron sheet thatched stone build house in Garsen, Tana River Delta. The housing type has influence on mosquito- human interractions which has effects on transmission rates. More fed and infected mosquitoes are found in houses with holes which allow mosquitoes seeking for blood meal get access into the houses.

6.3.6: Samples analysis

6.3.6.1: Mosquito identification and dissection

Mosquitos' collection in Busia- Matayos was done between March, 2020 for 15 days during the long rains which falls in March-June. In Tana River, collection was done between April, 2019 for 15 days during the long season that falls in

March- May. Female mosquitoes are the only blood suckers and known for disease transmission. Male mosquitoes do not feed on blood and thus together with other collected arthropods were desposed, females were selected, counted and preserved on silica gel for further processing. Mosquitoes were identified morphologically on a standard dissecting microscope and classified according to taxonomic identification keys (Gillett and Coetzee, 1987; Rattanarithikul, 1982). Mosquito dissection in search of microfilariae was performed

on 10% of mosquitos' daily collection. This was done immediately after killing the mosquitoes to ensure that any live microfilaria was observed and identified. Mosquitoes were placed individually on drop of saline solution on microscope slide. The head, thorax and abdomen were separated using dissecting pins and viewed under X10 magnification power of a dissecting microscope to confirm presence of any larvae stages (L_1, L_2 and L_3) as described by Leemingsawat *et al.* (1987). Parasite larvae stages identification was done as per observable characteristics as described by Chandler and Read (1969); L_1 is sausage shaped and immotile, L_2 is short with slow motility and L_3 is long, very motile and is the infective stage.

6.3.6.2: Immunochromatographic test by Alere™ Filariasis Test Strip

Seventy-five (75µl) of fingerprick blood was picked with micropipette provided in the package and placed on the FTS sample pad to detect circulating filarial antigens. For FTS positive cases, additional 4 Ml veinous blood was collected and taken to KEMRI- filariasis laboratory for further analysis by molecular assays described in chapter 3.

6.3.6.3: Mosquito and blood samples DNA extraction, PCR and LAMP Assays

Mosquitoes of the same species and from the same collection village were pooled into pools of a maximum of 20 mosquitoes into well labeled 1.5ML eppendorf vials. They were grinded in grinding buffer as per procedures described in chapter 3, section 3.4. Blood samples DNA extraction was done for every patient individually. Each extract was amplified separately by PCR and LAMP assays for validations. Detection of the amplicons were done by agarose gel electrophoresis and also compared by 1:10 SYBR green 1 dye.

6.4: Results

6.4.1: Participants' demographic data analysis during sampling for assays validation

Differential diagnosis was done on observable chronic cases of elephantiasis, hydrocele and patient's infection history. For this objective, a total of 387 field collected samples were used for validating the LAMP kit, 262 participants were recruited from Matayos in Busia and 125 from Tana River Delta. Descriptive analysis of the study population was done.

6.4.1.1: Age distribution of the recruited participants

Figure 6. 3 and Figure 6.4 shows age distribution withing the study area of Matayos and Tana River respectively. Participants in this study were aged between 4 years and 88 years. For analysis, age grouping of 10 years' difference was done. Majority of the participants in Matayos were aged between 11-20 years, n=58(22.14%) while in Tana River Delta**,** majority of the participants were aged above 60 years, n=35 (28%). In Matayos, the least participating group was 21-30 years with n=26 (9.92%) while in Tana River Delta, the least group was 1-10 years with n=2 (1.6%).

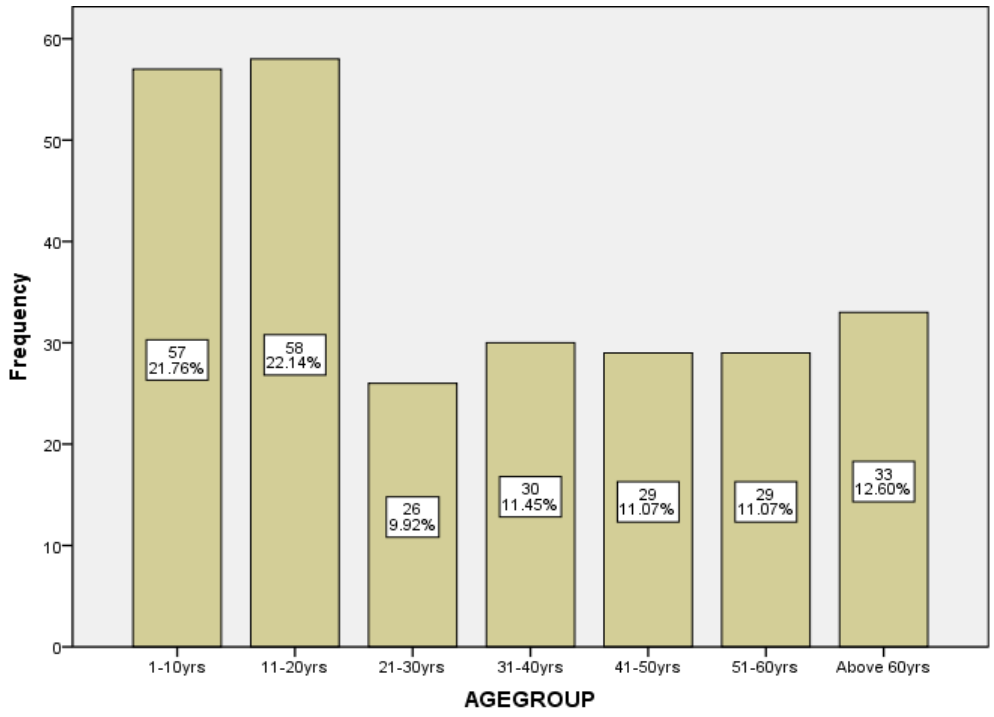


Figure 6.3: Participants grouping by age: (grouping difference of 10 years)- Matayos Busia study site

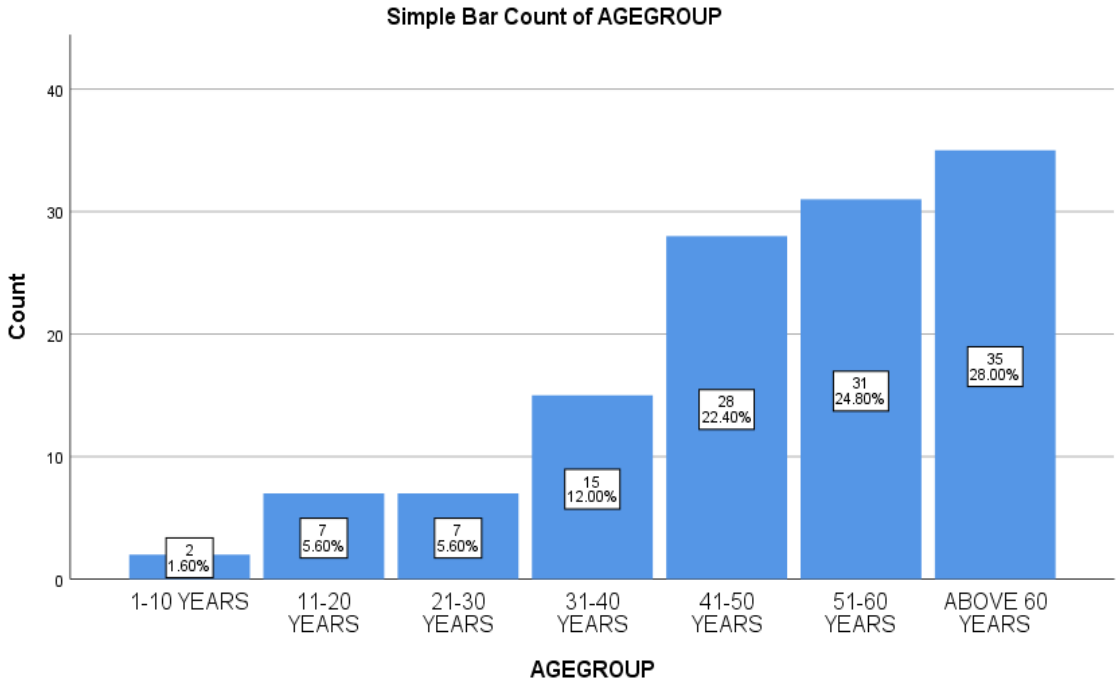


Figure 6.4: Participants grouping by age: (grouping difference of 10 years)-Tana River Delta

6.4.1.2: Sex representation of participants

Figure 6.5 shows sex representation in Matayos Busia with majority of the participants comprising of females n=160 (61.07%), while majority of the participants in Tana River Delta were males n=66 (52.80%) as shown in Figure 6.6.

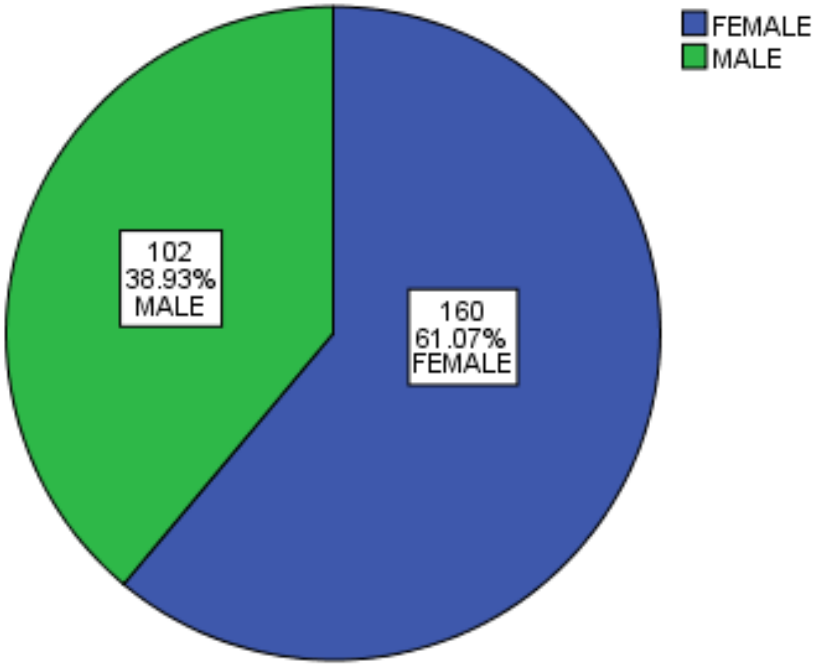


Figure 6. 5: Sex representation of the study participants in Busia Matayos

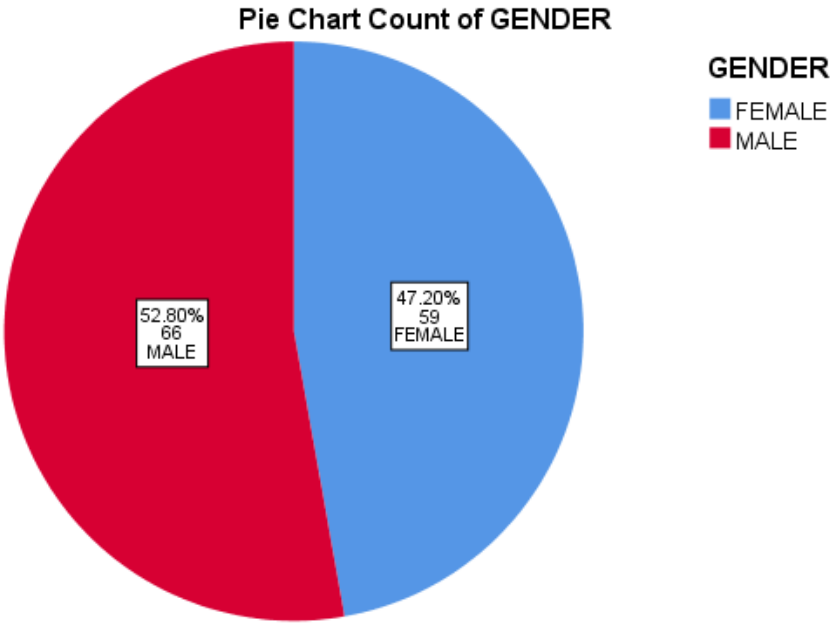


Figure 6.6: Sex representation of Tana River study population

6.4.1.3: Distribution of participants by villages

Table 6.1 shows distribution of participants by village in Matayos. Most of the participants in Matayos Busia were from Busende village n=64 (24.4%) and Mugweso n=60 (22.9%) with Nasira having the least participants n=7 (2.7%). Table 6.2 shows participants distribution in Tana River. Majority of participants in Tana River were from Odda Village n=57 (45.6% and Wema Village had the least n=15 (12.0%).

Table 6.1: Participants frequency by village / Community unit (CU) in Busia Matayos

VILLAGE	Sub-County	FREQUENCY (no)	PERCENT (%)
Bulandi	Matayos South	19	7.3
Emasiebia	Matayos South	22	8.4
Mugweso	Matayos South	60	22.9
Busende	Matayos South	64	24.4
Muyafwa	Matayos South	23	8.8
Murende	Matayos South	9	3.4
Lwanya	Matayos South	12	4.6
Nasira	Busibwabo	7	2.7
Nakhalina	Busibwabo	17	6.5
Busibwabo	Busibwabo	17	6.5
Nang'oma cu	Matayos South	12	4.6
Total		262	100

Table 6.2: Participants frequency by village in Tana River Delta

Village	Sub-County	Frequency(n)	Percentage (%)
Kajisten	Garsen	20	16
Maziwa	Garsen	17	13.6
Shirikisho	Garsen	16	12.8
Wema	Garsen	15	12.0
Odda	Garsen	57	45.6
Total		125	100

6.4.1.4: Categorization of participating population by signs and duration of the symptoms

Symptoms varied among the study participants. Some of the notable signs were swollen legs, either both or one leg(s) and scrotal swellings in males as shown in Figure 6.7 and Figure 6.8 respectively. Participants reported that they were experiencing signs for a varied period of time, ranging from less than a year to over thirty years.

Majority of the participants in both populations had no signs, Matayos Busia had asymptomatic participants n=192 and in Tana River n=57 as shown in table 6.3 & 6.4 respectively. In Matayos, most of the symptomatic participants n=37 had swollen leg/legs having lived for a period between 1-10 years with the conditions. Scrotal swellings were found in participants n=9 with 5 of them having lived with the condition for a period between 1-10 years and 1 person had the swellings for over 30 years. Other symptoms noted were painful and sensitive legs lasting for a period of 1-10 years as indicated in table 6.3 below. Swollen leg/legs were found in n=47 participants in Tana River Delta with n=23

having lived with the disease for about 1-10 years. Those with scrotal swellings were n=9, whereby n=4 patients lived with this condition for over 11 years. Characterization of participants signs and symptoms in Busia is as shown in table 6.3 while those in Tana River are as described in Table 6.4.

Table 6. 3: Signs and symptoms specified by period of time- (10-year difference); Matayos Busia Population

Period of Infection (Years)	No signs	Swollen leg (s)	Scrotal Swellings	Painful leg(s)	Sensitive leg(s)
0	192				
1-10 years		29	5	7	14
11-20 years		6	2	2	
21-30 years			1	1	
Over 30 years		2	1		
Total	192	37	9	10	14

Table 6. 4: Signs and symptoms specified by period of time- (10-year difference); Tana River Delta Population

Period of Infection (Years)	No signs	Swollen leg (s)	Scrotal Swellings	Painful legs	Sensitive legs
0	59				
1-10 years		23	2	4	2
11-20 years		12	4		1
21-30 years		8		3	
Above 30 years		4	3		
Total	59	47	9	7	3

6.4.1.5: Signs and symptoms representation by different age groups

Figure 6.7 and Figure 6.8 show different age groups presenting different symptoms in Matayos and Tana River Delta, majority of the participants had no signs across all the age groups. Swollen legs were high in age group 31-40 in Matayos while this condition was high among age group 51-60 in Tana River Delta. Scrotal swelling was high in age group 51-60 in Matayos but it was high in age group above 60 years in Tana River. Painful and sensitive feet was found to be high with the age group above 51 years of age in both

counties.

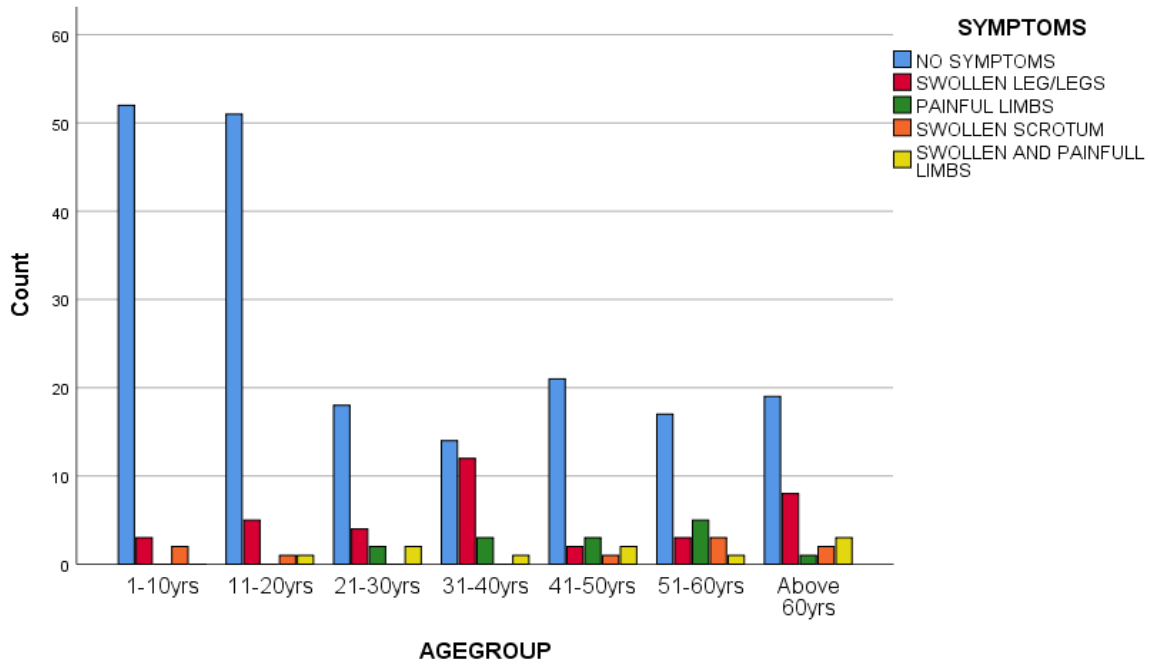


Figure 6.7: Bar graph representation of signs and symptoms by age group in Matayos population

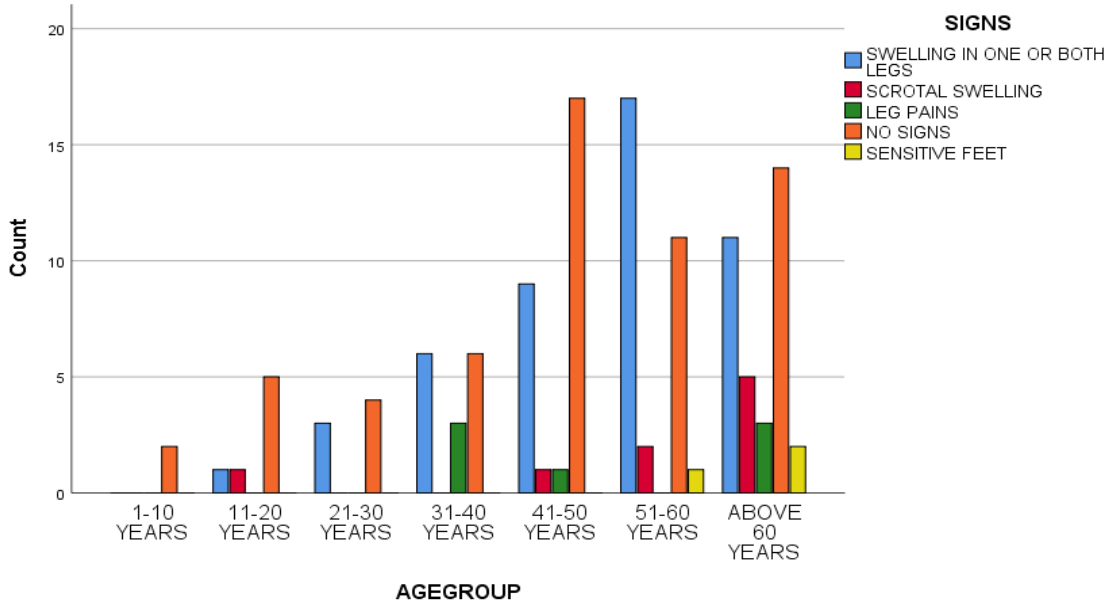


Figure 6.8: Bar graph representations of signs and symptoms by age group in Tana River Delta

Figure 6.9 and Figure 6.10 shows analysis of symptoms by villages in Tana River and Matayos respectively. Majority of the participants from Odda had no signs n=41 (69.49%) and in Matayos, most of participants n=59 (30.73%) without signs were from. In Tana River, swollen legs were high in Kajisten n=18 (38.3%) and lowest in Shirikisho n=5 (10.64%).

Scrotal swelling was high in Maziwa n=4 (44.44%) and no scrotal cases found in Kajisten. Sensitive feet were found only in Maziwa and Wema villages n=1(33.33%) and n=2(66.66%) respectively. For Matayos' population, swollen legs were high in Busibwabo with n=9 participants representing 24.32% of all leg swellings in this study population. There were no leg swellings with Lwanya village population. Leg pain were high with Muyafwa population n= 4 (28.57%). Scrotal swellings were high in Muyafwa and Nakhalina villages n=29(22.22%) each, with no cases in Lwanya, Nang'oma CU Murede and Nasira Villages. Sensitive feet were high in Nakhalina village n=3(30.0%). (Figure 6.10)

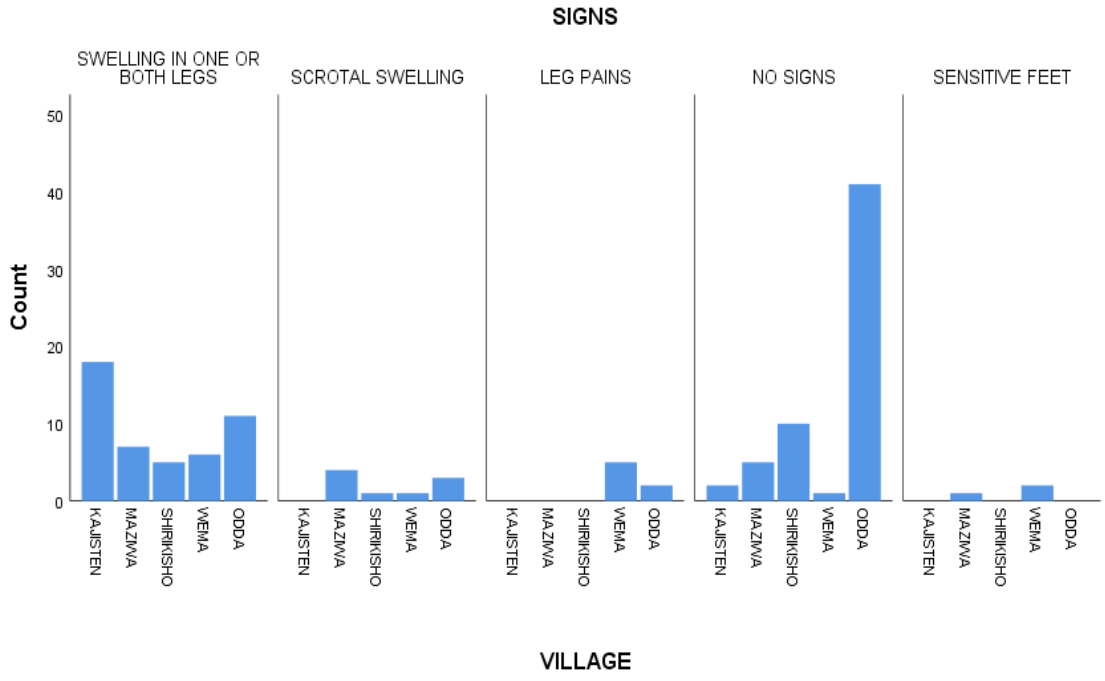


Figure 6.9: A bar graph of symptomatic representation per village in Tana River Delta Population

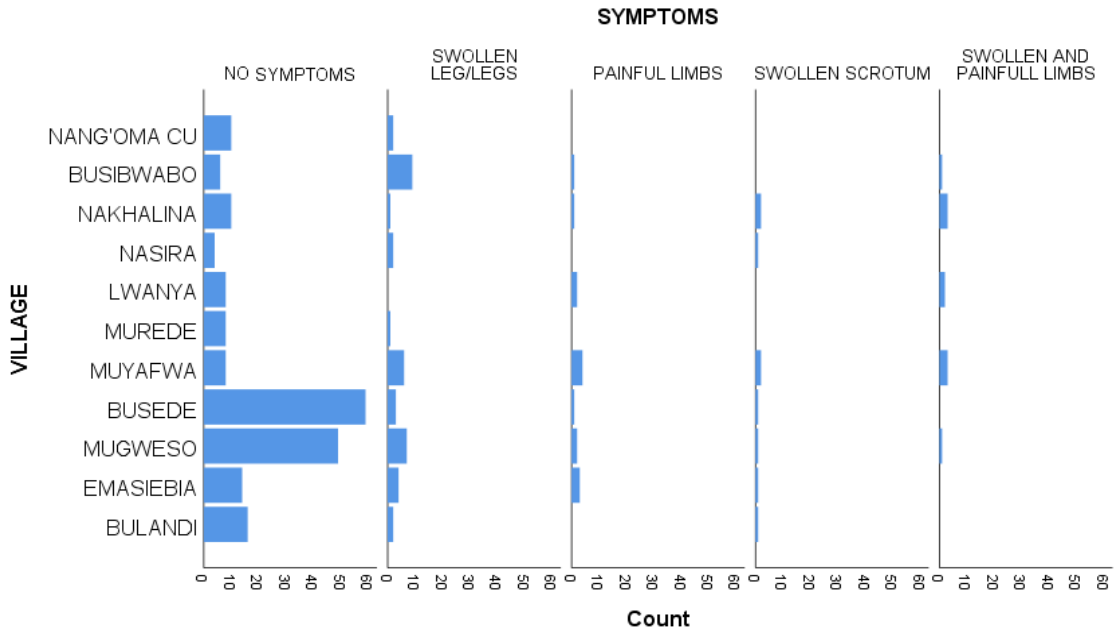


Figure 6.10: A bar graph of symptomatic representation per village in Matayos Busia population

6. 4.1.6: Prevalence of lymphatic filariasis in the study participants

In addition to observable signs, *W. bancrofti* infections prevalence within the study populations were determined by detecting circulating filarial antigens using Filarial Test Strip and by detecting presence of *W. bancrofti* DNA by PCR and LAMP in human population and vectors. Table 6.5 shows Matayos study antigenaemia prevalence of 17.6% (n=54) by FTS test and filarial DNA prevalence by both PCR and LAMP was similar, 7.3% (n=19). Tana River population antingemia prevalence was 17.6% (n=22) by FTS. Table 6.6 represents prevalences in Busia County, positive cases were 13, 15, 18 representing prevalence of 10.4%, 12% and 14.4% by PCR, LAMP and HDA respectively (Table 6.6).

Table 6.5: Prevalence of filarial infections in Matayos Busia study population by cPCR and LAMP assays

Test method	No. Negative (%)	No. Positive (%)	Total No. tested	Prevalence of infections (%)
FTS	208	54	262	17.6
cPCR	243	19	262	7.3
LAMP	243	19	262	7.3

The infection prevalences in Matayos were found to be 17.6 by FTS and 7.3 % by cPCR and LAMP. The same prevalences by PCR and LAMP showed a perfect agreement (K_{value} 1.0)

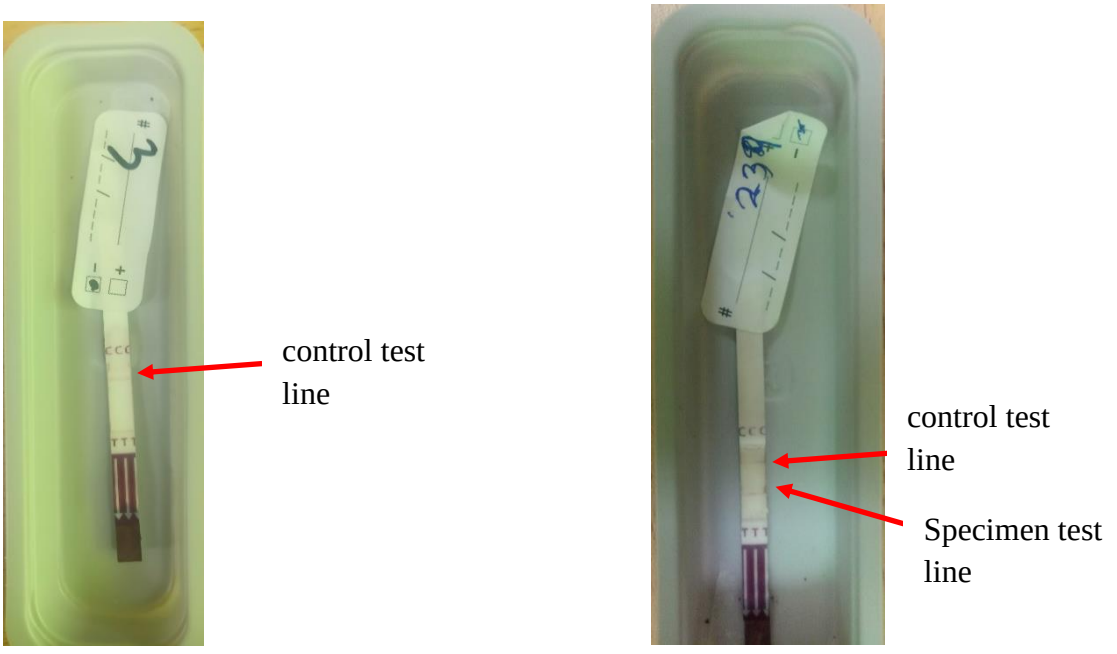
Table 6.6: Prevalence of filarial infections in Tana River study population by different detection methods

Test method	No. Negative (%)	No. Positive (%)	Total No. tested	Prevalence of infections (%)
FTS	103	22	125	17.6
cPCR	112	13	125	10.4
LAMP	110	15	125	12.0
HDA	107	18	125	14.4

The infection prevalence was found to be 17.6 by FTS and 10.4 % by cPCR, 12.0 by LAMP and 14.4 by HDA. The K_{value} for LAMP was 0.84 and that of HDA was 0.67.

6.4.1.7: *W. bancrofti* circulating filarial antige Test by Filarial Test Strip

All participants were tested for the circulating filarial antigen test by FTS as shown by figure 6.11 below.



(a) (b)

Figure 6.11: Image (a) showing a negative circulating filarial antigen test results (FTS) and image (b) showing positive filarial antigen test (FTS). The pink line is only seen on the control line and with no indication on the test line hence negative results while in image (b) there are two pink lines on the test and the control lines hence positive results.

6.4.1.8: *W. bancrofti* DNA amplification by PCR and detection on agarose gel electrophoresis

Figure 6. 12 shows amplification of W. bancrofti DNA by PCR and detected on agarose gel electrophoresis. Positive bands align themselves to 200bp band on 100bp molecular marker. Tbale 6.7 shows amplification of field collected specimens from Matayos and from Tana River by cPCR and LAMP methods for LAMP assay validations.

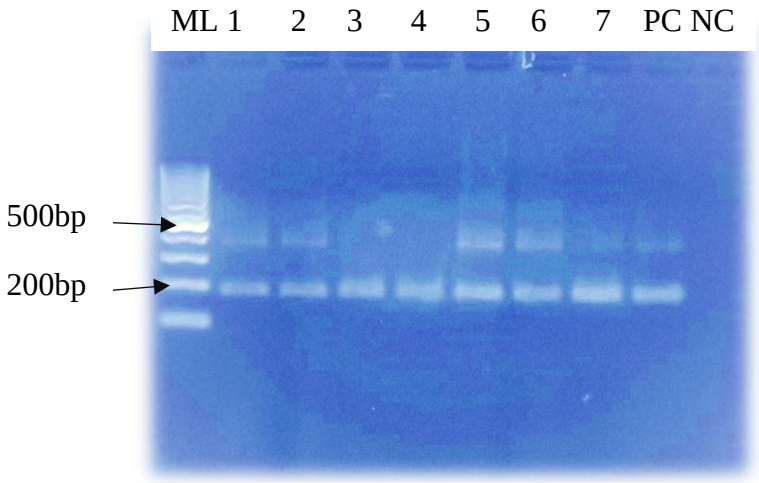


Figure 6.12: Image of PCR products on 2% agarose gel electrophoresis represented by bands size of 188bp, the 18S rRNA targeted region

ML represents Molecular size standard marker (100bp), Lanes 1-7 are positive specimen, Lane PC is *W. bancrofti* DNA positive control and Lane is NC is the negative control.

Table 6.7: Field validation of LAMP assay in Matayos Busia & Tana River Delta

LAMP TEST	PCR TEST		TOTALs
	Positive	Negative	
Positive	29 (a)	5 (b)	34
Negative	3 (c)	350 (d)	353
TOTALS	32	355	387

Sensitivity= a/a+c =29/32 = 91%, **Specificity =** d/b+d= 346/351 =99%

Kappa statistics = 0.87 (Near Perfect agreement).

6.5: Descriptive analysis of mosquito collected from the study areas

Table 6.8 and Table 6.9 are descriptive analysis of indoor and outdoor collected mosquitoes in Matayos and Tana River respectively. Five (5) villages were randomly selected for mosquito trapping in Matayos Busia and in Tana River Delta study sites. In the current study, 3321 mosquitoes were collected, of this 1305 were obtained from Matayos and 2016 from Tana River Delta. In all the genus, majority of the mosquitoes from Matayos were collected from indoors n=1254 (96.09%) and outdoor collection was n=51 (3.91%) (Table 6.8). *Culex species* had the highest outdoor collection n=46 (3.52%) as compared to the others. A total of 1722 representing 85.42% mosquitoes were from indoor collection in Tana River while n=294 (14.58%) were from outdoor trapping (Table 6.9). On identifications, mosquitoes of genus; *Anopheles* species, *Culex* species, *Aedes* species, *Coquilletidia* species were obtained from Matayos. *Anopheles* species were the majority with n=832 representing 63.75% of the total collection, while *Coquillettidia* species were the least n=15 (1.15%) (Table 6.8).

Table 6.8 Mosquito genera collected in Matayos

Mosquito genus	Site of collection		Total
	Indoor count/percentage	Outdoor count/percentage	Genus collected count/percentage
Anopheles species	829 (63.52%)	3 (0.23%)	832 (63.8%)
Culex species	387 (29.66%)	46 (3.52%)	433 (33.2%)
Aedes species	24 (1.84%)	1 (0.08%)	25 (1.9%)
Coquillettidia species	14 (1.07%)	1 (0.08%)	15 (1.1%)
TOTAL	1254 (96.09%)	51 (3.91%)	1305 (100%)

Five (5) genera of mosquito were collected from Tana River which included *Anopheles, Culex, Aedes, Mansonia* and *Filcabia* species. Out of the 2016 mosquito collected, *Culex* had the highest catch of n= 876 representing 43.5% and *Filcabia* species had the least catch n=32 (1.6%) (Table 6.9) below. *Culex* species had the highest outdoor collection n=117 (8.78%) compared to the rest of the genera obtained outdoor.

Table 6.9 Mosquito genera collected in Tana River

Mosquito Genus	Site of collection		Totals
	Indoor Count/ Percentage	Outdoor Count/ Percentage	Genus Collection Count/Percentage
Anopheles species	671 (33.28%)	19 (0.94%)	690 (34.2%)
Culex species	699 (34.67%)	117 (8.78%)	876 (43.5%)
Aedes species	254 (12.6%)	61 (3.03%)	315 (15.6%)
Mansonia species	74 (3.67%)	29 (1.44%)	103 (5.1%)
Filcabia species	24 (1.19%)	8 (0.4%)	32 (1.6%)
TOTAL	1722 (85.42%)	294 (14.58%)	2016 (100%)

6.5.1 Feeding status of the mosquitoes collected

Figure 6.13 shows mosquito classified according to feeding status for Busia collection, either having taken blood meal (Fed) or not taken blood meal (unfed). Out of 1305 mosquitoes collected from Matayos, majority of them were unfed n=1140 (87.36%) while the fed mosquitoes were 165 (12.64%). It is important to note that the source of blood meal was not determined in this study.

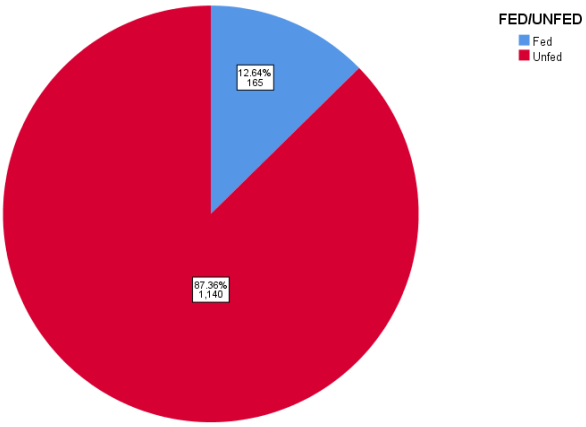


Figure 6.13: A pie chart of mosquito feeding status in Busia Matayos

Figure 6.14 shows mosquito feeding status in Tana River, where by 1730 mosquitoes were unfed representing (85.81%) while 286 (14.19%) were fed.

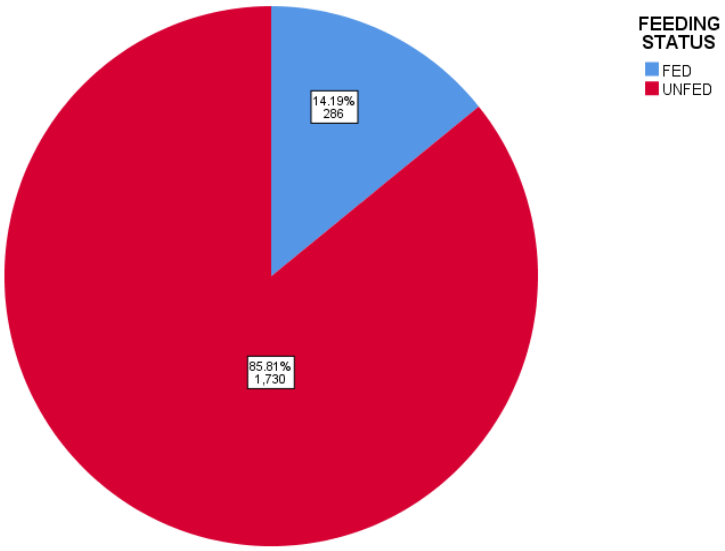


Figure 6.14: A pie chart of mosquito feeding status from Tana River Delta

6.5.2: Mosquito distribution per village of collection

Figure 6.15 show mosquitoes' distribution in Matayos villages, Mugweso village had the highest catch n=546 (41.87%) while Emasiebia had the least n=16 (1.23%). Mosquito distribution in Tana River collection villages is shown in Figure 6.16. Majority of the mosquitoes caught were from Maziwa village with n= 627 (31.10%) while Odda village had the least catch 314 (15.58%). (Figure 6.16).

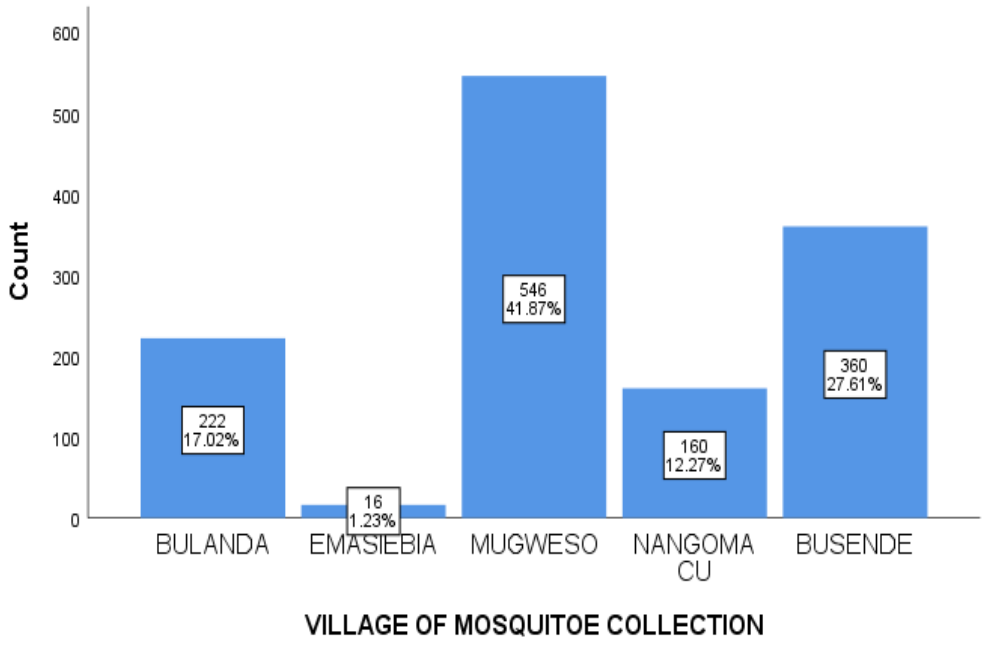


Figure 6.15: A bar graph of mosquito collection per village in Matayos

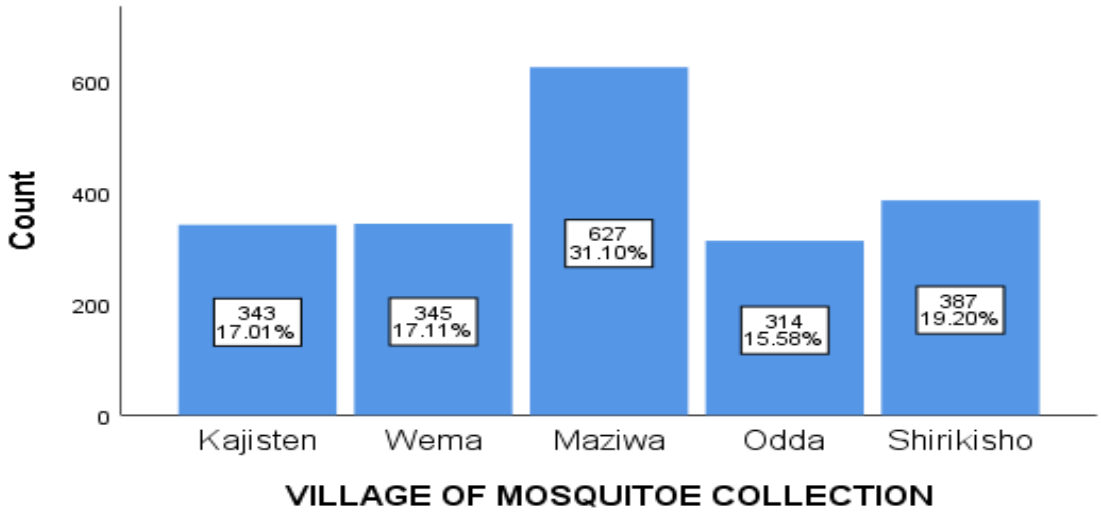


Figure 6.16: A bar graph of mosquito collection per village in Tana River

6.5.3: Mosquito composition in the collection villages

Composition of the collected mosquitoes was annalysed per species and recoded in Figure 6.17 for Tana River and Figure 6.18 for Matayos. *Culex* species were the most abundant in all the collection villages in Tana River except Shirikisho village which had *Anopheles* species as the most abundant. *Filcabia* species were the least collected in each village. Most of *Aedes* species were collected from Kajisten village (Figure 6.17). In Matayos the most abundant mosquito species were *Anopheles* species, which were more prevalent in all villages apart from Emasiebia which had *Culex* species as the most abundant. *Coquillettidia* were only found in Nangoma CU and Busende villages (Figure 6.18). There were no *Mansonia* or *Filcabia* species in Busia collection neither were there *Coquillettidia* species found in Tana River Delta.

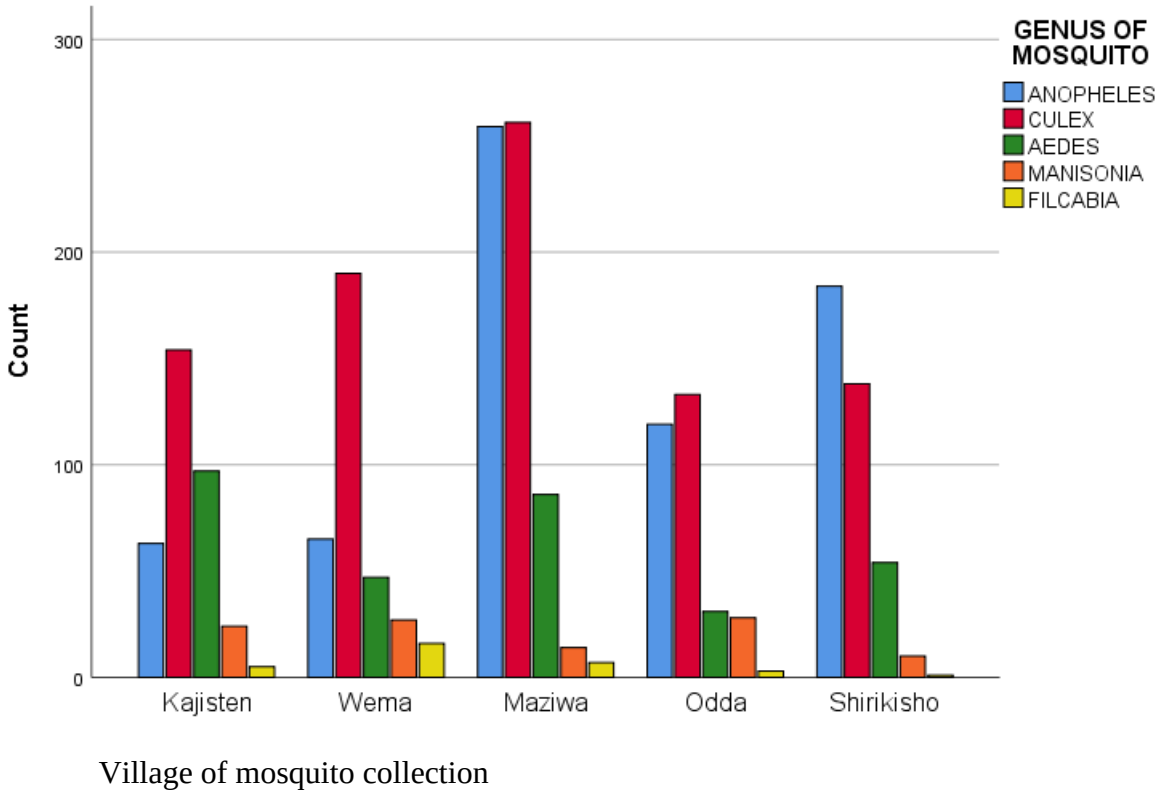


Figure 6.17: Mosquito genus collected per village in Tana River

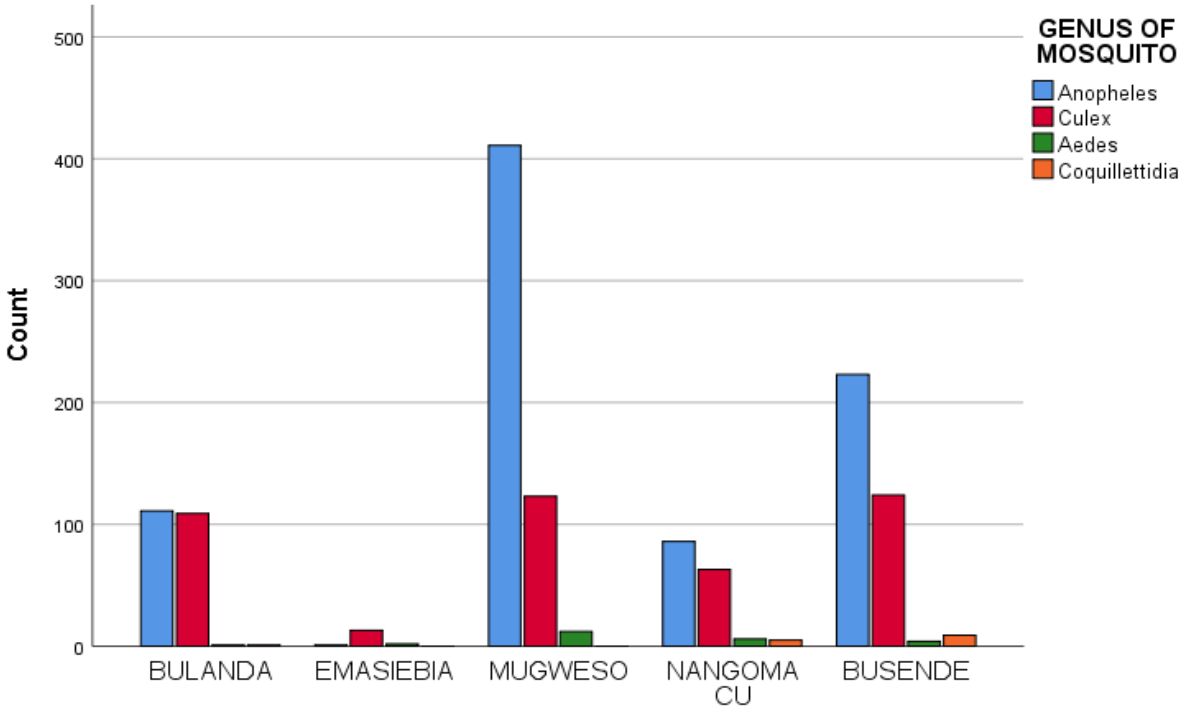


Figure 6.18: Mosquito genus collected per village in Matayos

6.5.4: Mosquito infection and infectivity rate

Mosquito infection rates and infectivity rates were determined by dissecting the mosquitoes in search of microfilaria (L_1-L_3). Mosquitoes were pooled as per species and village of collection to determine the Minimum Infection Rate (MIR). Polymerase chain reaction was performed on mosquito pools. Table 6.10 represents mosquito dissection results which were used to calculate MIR in Tana River.

Table 6.10: Dissection of mosquitoes collected from Tana River

Collection Area	No. of mosquitoes dissected	No. Infected (with larvae)	Not infected (without larvae)	PCR detection (DNA detection on pools)
Tana River	202	2 larvae with 1 (L_3)	2014	1
Busia	131	0	131	2

Tana River infection rate; No. of larvae/No. of mosquitoes dissected

2/202= 0.99% ~0.01

Infectivity rate; No. of L_3/ No. of mosquitoes dissected

1/202= 0.49% ~0.005

There were no larvae found in dissected mosquitoes from Matayos mosquitoes thus, infection and infectivity rates in Matayos were both zero (0).

6.5.5: Mosquito pooling for DNA detection and determination of the Minimum Infection Rate (MIR)

Table 6.11 and 6.12 shows mosquito pools per village from Matayos and Tana River respectively. On pooling the mosquitos per species and per village of collection, 78 pools were obtained from Matayos and 118 pools from Tana River. DNA extraction and detection was done on all the 196 pools. One pool from Tana River and two pools from Busia had *W. bancrofti* DNA as detected by PCR. To determine mosquito infection prevalence in pools, MIR was estimated by determining the number of infected mosquitoes per 1,000 represented as [number of positive pools/total specimens tested] x1000 (Biggerstaff, 2003). This method for estimation of infection assumes that only one mosquito is infected in a pool, however the limitations by this method are that during disease epidemics when the infection rates are high in the vectors, disease transmission rates may be under-estimated. In this study, the rate of mosquito infections with filarial parasites are generally low, thus this method was suitable for the estimations.

Figure 6.19 shows PCR amplification of *W. bancrofti* microfilariae DNA in mosquito pools.

Figure 6.20 shows LAMP assay amplification of *W. bancrofti* DNA in mosquito pools.

minimum infection rate (MIR) in Matayos;

(2/1305) 1000 = 1.532

minimum infection rate (MIR) in Tana River;

(1/2016) 1000= 0.496

Table 6.11: Mosquito pools per species per village in Matayos

Village of collection	Mosquito species								PCR	Total Pools
	Anopheles Species		*Culex* Species		*Aedes* Species		*Coquillettidia* Species			
	Count	Pools	Count	Pools	Count	Pools	Count	Pools		
Bulanda	111	**6**	109	**6**	1	**1**	1	**1**	1	**14**
Emasiebia	1	**1**	13	**1**	2	**1**	0	0	0	**3**
Mugweso	411	**21**	123	**7**	12	**1**	0	0	1	**29**
Nangoma CU	86	**5**	63	**4**	6	**1**	5	**1**	0	**11**
Busende	223	**12**	124	**7**	4	**1**	9	**1**	0	**21**
TOTAL Pools		45		25		5		3	2	**78**

Table 6.12: Mosquito pools per species per village in Tana River Delta

Village of collection	Mosquito species										PCR	Total
	Anopheles species		*Culex* species		*Aedes* species		*Mansonia* species		*Filcabia* species			
	Count	**Pools**	Count	**Pools**	Count	**Pools**	Count	**Pools**	Count	**Pools**		
Kajisten	63	**4**	154	**8**	97	**9**	24	**2**	5	**1**	0	24
Wema	65	**4**	190	**10**	47	**3**	27	**2**	16	**1**	0	20
Maziwa	259	**13**	261	**13**	86	**5**	14	**2**	1	**1**	0	34
Odda	119	**6**	133	**7**	31	**2**	28	**2**	3	**1**	1	18
Shirikisho	184	**10**	138	**7**	54	**3**	10	**1**	1	**1**	0	22
TOTAL Pools		**37**		**45**		**22**		**9**		**5**	**1**	**118**

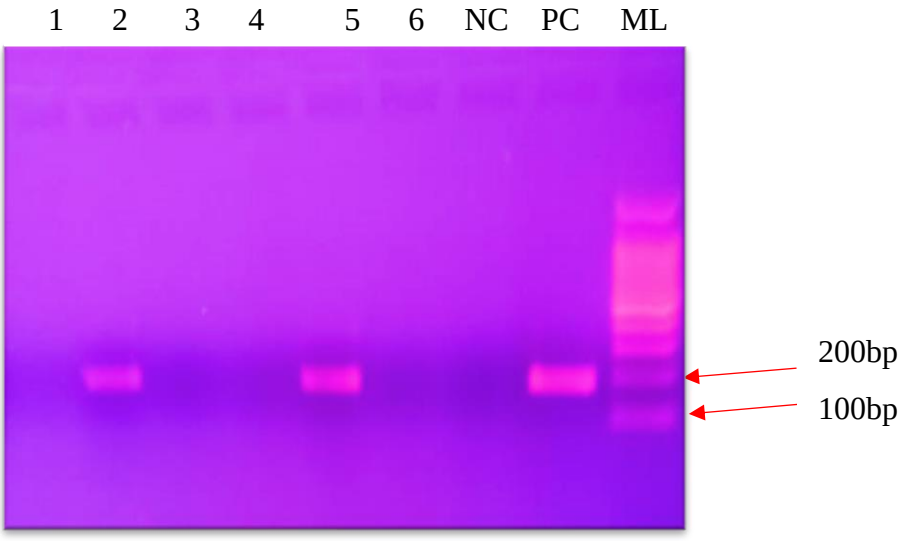


Figure 6.19: Image of PCR amplification product of *W. bancrofti* DNA from vectors collected from the study area.

Wells: 1, 3, 4, & 6 contained uninfected mosquitoes; wells: 2 & 5 were mosquitoes infected with *W. bancrofti* DNA. NC represents Negative control, PC is Positive control containing *W. bancrofti* DNA and ML is a molecular size ladder (100bp).

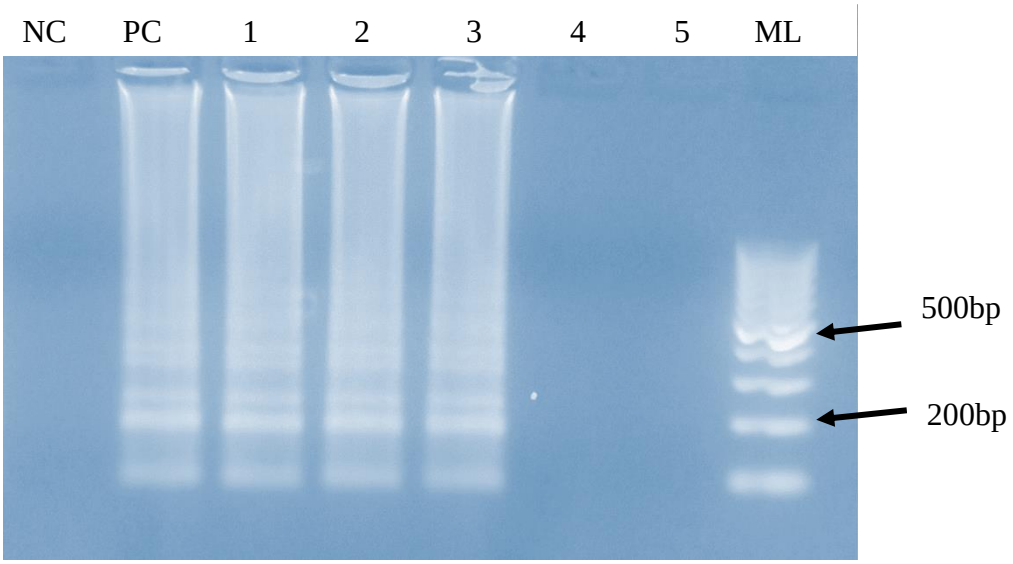


Figure 6.20: LAMP products of W. bancrofti microfilariae DNA in the vectors collected from study area.

Wells: NC is Negative control, PC is Positive control containing *Wuchereria bancrofti* DNA, 1 & 2 wells contain mosquitoes infected with *W. bancrofti* DNA from Matayos Busia while well 3 contains mosquitoes infected with *W. bancrofti* DNA from Tana River, wells 4 and 5 are uninfected mosquitoes, ML is a molecular size ladder (100bp).

6.5.6: Evaluation of LAMP sensitivity and specificity for detection of *W. bancrofti* in mosquito vectors

In this study, three pools of mosquitoes tested positive for *W. bancrofti* DNA with PCR and LAMP assays. For proper validation of LAMP assay, genomic DNA (gDNA) of the mosquito pools were spiked with *W. bancrofti* DNA from control cultures for mosquitos' infection simulation. Five microlitre (5µl) of *W. bancrofti* positive control was added to 200µl of the mosquito DNA extract, equivalent of 5 copies of DNA in one microfilaria. The concentration of extracted DNA from the mosquitoes was measured on a NanoDrop™ 2000 spectrophotometer (Thermo Scientific) and serial dilutions carried out from 0.1 fg to 10 pg to test the detection limit of *W. bancrofti* DNA in vectors. Specificity of LAMP

assays were determined by adding 5µl of positive control which included *Echinococcus granulosus, brugia malayi*, *Anopheles gambiae*, *Schistosoma mansoni*, *Plasmodium falciparum* and *Trichuris trachura* to 200µl of mosquito DNA extracts. DNA extraction, LAMP and PCR amplifications were done as detailed in chapter 3 section 3.4. LAMP for mosquitoes was able to test up to 10^{-4} slightly lower concentration to that of human blood and PCR. The specificity was the same since only the extracts containing *W. bancrofti* were amplified. LAMP results were as shown in Figures 5.5 and 5.6 for sensitivity and specificity respectively. This was a clear indication that LAMP assay validated in this study are suitable for monitoring *W. bancrofti* transmission in vectors.

6. 6: Discussion

The results presented in this study focused on validating LAMP assay for diagnosis of *W. bancrofti* in a field set up. The samples were obtained from two different field settings. Tana River Delta, an endemic area in coastal region where MDA has been taking place since 2011 (Njenga *et al*., 2017) and Matayos Busia where there are no MDA activities in place. Matayos Constituency in Busia County was selected for field validation of the kit since there were cases reported in the region but no proper endemicity data available. Human populations were screened for circulating filarial antigens by Alere Filariasis Test Strip (FTS) and presence of filarial DNA detection by Polymerase Chain Reaction and LAMP. Mosquitoes were collected, identified and dissected for presence of filarial larvae (L_1-L_3) and then amplified for presence of *W. bancrofti* DNA determination.

Descriptive statistics of the participants were analyzed by IBM SPSS statistical software version 23. Participants above 60 years of age were more frequent in Tana River (Figure 6.4). Observations show that older person's have access to more health programmes and in most cases have realized the benefits of participating in such campaigns as majority have different health related problems. Since the disease takes a number of years to manifest to clinical symptoms, older adults have suffered more than the young age groups. These observations were similar to reports by Krentel and colleagues (2016) and Talbot and colleagues (2008). In contrary to Tana River, majority of people who turned up for screening in Matayos were in the age bracket 11-21 years representing (22.14%) of the screened population (P=0.001). This is because this age group was found to be more enthusiastic to participate in the study being the first one of its kind in the study population. Kagai and colleagues in 2008 made similar observations which suggested that, whenever lymphatic filariasis survey is to be carried out, age group 11-20 years old are the most

appropriate participants to accurately represent infections in the community. This group had 9.2% detectable infections though they had no signs, confirming the suggestions by Kagai and colleagues that this is the most appropriate group for assessing infection rates in endemic regions. Infections take long period of time to manifest to chronic stages and thus victims remain asymptomatic for many months or even years despite the fact that they are the major carriers of circulating microfilaria. There were more males than females in Tana River populations while in Matayos, females were more than the males. This is due to the fact that different ethnic groups have different social and cultural practices. In Tana River females have to seek for permissions to take part in any medical exercise, the fact that most males are away carrying out day to day activities in search of livelyhood for the family, females miss the opportunity to take part as their male counter parts are not always present at homes to permit them. These findings were comparable to those of a study by Njomo and colleaques (2020) who found out that female could not engage in any MDA exercise without their husband's permissions together with children under 18 years of age. In contrary, females in Matayos could take part in the exercise without males' permission, hence the large number of females than males in Matayos population. This could also be because females are much available and can easily be found at homes than men who majority of them are busy away from the villages. Also, most of men refuse to take part in such studies, especially the ones with hydrocele conditions shun from testing due to the stigma associated with it. Busende Community in Matayos had the most participants n=64 (24.4%), while Odda in Tana River had the highest participants n=57(45.6%), this could be attributed to the time taken in each unit and the mobilizing ability/awareness of the Community Health Volunteer (CHV) involved among other factors. Villages which had witnessed more cases were more willing to take part in the study as compare to those with few or no cases.

Lymphatic filariasis manifest in different signs; asymptomatic and symptomatic cases which include acute and chronic forms. The chronic conditions present in the forms of elephantiasis, lymphedemas and hydrocele cases. In the current study, majority of the participants did not show any signs for both Matayos and Tana River Delta populations. Most of the people with observable signs in Matayos reported that they have had the signs for a period between 1-10 years but only a few with swollen legs and scrotal swellings have had the disease for over 30 years (Table 6.3). Chronic forms of this disease cause huge social and economic burden to the individuals, relatives and the health care facilities, as reported by Wynd and colleaques (2007). In this study, participants with chronic stages of the disease reported that they had lived with the disease even for over thirty years and there was a high significance between the duration of symptoms and the age group (df =6, f= 5.228 Sig.0.001) and (df = 6, f = 6.558 and Sig. 0.001) for Busia and Tana River respectively. This suggests that, even during the initial mapping in Kenya (1998-1999) (Wamae *et al.,* 2001), there were few cases that did not meet the threshold of >1% prevalence stipulated by WHO for an area to be considered as endemic and have control measures put in place. With time, transmissions take place from the few cases which spread to a large population and developed to chronic stages if left untreated. Despite the fact that the disease progression to clinical signs take many years to manifest and is of major public health concern, it still remains neglected in this region.

In Tana River, majority of participants with scrotal swellings reported that they have had the disease for a period of 11-20 years, while majority of those with swollen legs, painful legs and sensitive legs have had the problem for a period of 1- 10 years. Swollen legs were high in Kajisten with scrotal swellings high in Maziwa in Tana River, while in Busia population swollen legs was high in Busibwabo and Nakhalina had the highest scrotal swellings. In Tana River community, scrotal swellings were high with age group of above

60 years with non reported in age groups between 21 to 30 and 31 to 40 years. Similarly, there were no scrotal swellings observed within the age groups 21-30 and 31-40 during the study period in Matayos despite the fact that community elders had reported some cases within these age groups. This is associated with the stigma it causes leading to many youths of this age groups to shun from revealing their conditions. Scrotal swellings were high within the 51-60 years' age group and also presented in age group 1-20 years. Locals believed that the disease was witchcraft or hereditary disease (referred as family disease) and this made some of the victims not to seek for medical attention even when capable of paying for hydrocelectomy procedures. There is need to create awareness of the disease transmission and a manifestation for the community to be aware that elephantiasis is an infectious disease transmitted by mosquitoes and is preventable and treatable at early stages. From this study, it was evident that Matayos and Busibwabo wards in Busia County have been experiencing filarial infections transmissions in the population for over 20 years. It was noted with great concern that there were no records of filarial infections found at Matayos sub-County referral hospital due to lack of detection capacity (Test Kits), but they clearly mentioned that the suspected cases were referred to KEMRI- ALUPE, Busia. Some individual cases that were referred to KEMRI- ALUPE Busia, reported that they were tested by microscopy of night collected blood and medications prescribed for them (Nancy, Personal communication). Majority of them reported that they would feel better on medications but the swellings would only reduce and never disappear completely, eventually they would give up from seeking for medical help because of the permanent swellings. It is important to mention that chronic stages of filariasis are not treatable, this calls for early screening and treatment. It is unfortunate that patients only seek for medical attention when the disease has already manifested to chronic stages and at this point, only morbidity management and disability prevetion interventions can help.

In this study, LAMP assays validation confirmed that the assays can be used to replace PCR methods in a field set up. During validation of the LAMP kit, 387 samples were used from two different study sites. The antigenaemia prevalence found during this study in Tana River was lower than that initially found before the start of MDA programmes by Kagai and colleaques (2008). This indicates that MDA programmes have led to decreased prevalence. However, comparing to studies by Njenga *et al*., (2017), the prevalence in this study is higher and this could be due to resurgence of the infections despite the fact that this study used a smaller population. These observations may be due to MDA non-compliance and low coverage as it was previously observed by Njomo and colleagues (2021) and by Boyd and colleagues (2010). During this LAMP assay validation study, sensitivity and specificity of LAMP was at 91% and 99% respectively which was much comparable to that of PCR with a Kappa value of 0.87 showing a near perfect agreement as found in publications by Kinyatta and colleaques, (2021) for this study.

Five mosquito genera were obtained from the collection sites in Tana River (Figure 6.17) while there were only four genera found in Busia (Figure. 6.18). *Culex* species were more prevalent in Tana River County which is the main vector of lymphatic filariasis in urban and rural areas of coastal Kenya as reported in studies by Kinyatta and colleaques (2018) and by Mwandawiro and colleaques (1997). *Anopheles* species were more abundant in Busia. Busia County in Kenya is known to have high malaria prevalence transmitted by *Anopheles* species of mosquitoes (Edward *et al.*, 2020). Each village had different number of mosquitoes collected suggesting that there were differences in ecological factors in different villages sampled. This was noted as more mosquitoes were obtained from areas near water bodies. The highest catch was in Maziwa area in Tana River while Mugweso village had the highest catch in Matayos. A significance difference on the mosquito species obtained from the collection villages was observed as given by (df = 4, f = 31.720 p =

0.001) for Tana River mosquitoes and (df = 4, f = 18.163 and p =0.001) for Matayos. These two study sites were found to have a lot of water bodies surrounding the village as compared to other collection sites. Mosquito density increases during the wet season due to the availability of mosquito breeding sites (Evans *et al*., 1993). Uncontroled urbanization and poor sanitation amenities have contributed to increase in mosquito breeding sites for vectors of filarial infections. *Culex* species breed are found to breed in wet pit latrines while *Mansonia* species attached on submerged vegetations (Mwandawiro *et al*., 2007; Rajenran *et al*., 1989).

Indoor and outdoor mosquito sampling showed a significant difference (df=1, sig. 0.001 at 95% CI), Table 6.8 for Matayos and Table 6.9 for Tana River. Generally, majority of the mosquitoes were obtained indoors for both study sites. For the outdoor collection, *Culex* species were the most abundant for both study sites. Mosquitoes have different host seeking behaviour and resting behaviour. *Anopheles* mosquitoes seek for blood meal and rest indoors and thus many of them were trapped from indoors. *Culex* feed indoors and rest outdoor and that is the reason why a good number were caught from outdoor trappings. There was a correlation between mosquito genera collected and the areas of trapping (df=1, f= 12.171 sig. 001) for Tana River and (df=1, f=56.286 sig. 0.001) for Matayos.

The nature of houses and the housing materials influenced mosquito densities collected. Houses in Tana River were either made of makuti/grass thatched on roofs and walls or block walled and galvanize iron sheet roofs with windows fitted window screens. In Matayos, the houses were made of mud walled thatched with grass/makuti or block walled and galvanize iron sheet roofs with windows having window screens. There were few mosquitoes obtained from houses build with blocks, iron sheet roofs and fitted with window screens. During mosquito collection, Odda village in Tana River had few mosquitoes collected simply because houses in this village were build using blocks and

iron sheet for roofing. It was also observed that the windows had window screens thus mosquitoes could not easily enter into the houses. A lot of mosquitoes were obtained from mud-walled grass thatched houses, majority of which had open windows for ventilations and many other openings (holes) into the houses.

Filarial infection rate in vectors is an important parameter in determining transmission indices. Infection rates refers to the presence of any larvae stages within the mosquito body parts while infectivity rate is the presence of L_3, which is the infectious stage. Different mosquito species have different capacity of carrying the larvae to infection stages and thus not every mosquito carrying L_1- L_2 has the vectorial potential of transmitting filariasis (Kinyatta *et al.*, 2011). In the current study, infection and infectivity transmission rates of the collected mosquitoes were 1% and 0.5% respectively in Tana River. This was different from what was observed in studies by Njenga *et al.*, (2017) who found that there were no infected mosquitoes by dissection in mosquitoes collected from Tana River. There were no infected mosquitoes in Matayos by dissection but there were two positive pools by both PCR and LAMP. Matayos had a minimum infection rate (MIR) of 1.532 which was higher to that of Tana River (0.496). The extremely low levels of transmissions of lymphatic filariasis in the vectors from Tana River are linked to use of different vector control measures by the community and decreased prevalence in the humans due to MDA interventions. For instance, it was observed that the use of Insecticide Treated Bed Nets (ITN), which was implemented by the malaria control programme is widespread in both study areas as it was observed by Njomo *et al.*, (2021) in Kilifi and Kwale counties which are filariasis endemic areas. In Tana River, people practice traditional methods of controlling mosquitoes such as use of firewood smoke in both outside and inside the houses as a means of preventing mosquitoes from getting into the houses. Importantly, use of LLIN and deworming programmes have significantly contributed to reduced lymphatic

filariasis infection despite the irregular implementation of MDA as it was found by Njenga *et al*., (2017).

6. 7: Conclusions

In this study, evaluation of LAMP assays for detection of *W. bancrofti* DNA targeting 18S rRNA gene has shown that it is capable of detecting the parasites' DNA in humans and vectors. The assay was highly sensitive and highly species specific, as it did not detect DNA of other closely related filarial parasite such as *Brugia malayi* and non- filarial parasites.

High filarial antigenaemia prevalence found in the study sites calls for control and preventive mechanisms to be intensified in this region. Chronic cases such as lymphedemas and hydrocele which were so much evident in the study areas need morbidity management and disability prevention intervetions. However, these interventions have started in few Kenyan coastal endemic regions which needs to be intensified to cover the orher endemic regions. Hydrocele cases can easily be managed by surgical interventions with the affected people requiring financial support. Mosquito genus found in Matayos are vectors of both malaria and filariasis. With high disease prevalence in the population and mosquito vectors available, it is evident that the parasite is in circulation in the community and this warrants for control measures.

6.8: Recommendations

This study recommends use of LAMP assay for diagnosis of *W bancrofti* which is relatively low in cost and time effective to be captivated for mapping, monitoring and evaluating lymphatic filariasis control programmes.

Since this intitial study in Busia showed that filarial infections have been in circulation in the population for over 20 years, a large population needs to be screened to establish the

actual infection prevalence in whole of Busia County before mass drug administration is recommended. Mosquito prevention measures need to be strengthened to control transmissions. The County Government of Busia under the Universal Health Coverage should chip in to assist the community by providing filarial testing kits in hospitals for early diagnosis and treatment. Basic morbidity management and disability prevention measures to affected people should also be provided.

This study, found a possible resurgence of infections in Tana River despite the fact that annual MDA continues to be offered to the population and integrated control programmes should be intensified in these endemic regions.

CHAPTER SEVEN: GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1: General discussion

Manifestation of filarial nematodes ranges from asymptomatic infections to clinical chronic stages (Dietrich *et al*., 2019). There is lack of diagnostic tools in many clinical set up in endemic regions, thus the parasites are not routinely tested. Most of the regions rely on clinical manifestations which take long period of time (1-10 years) to manifest. Lymphedema and elephantiasis cases are not treatable and hydrocelectomy is the only way out to relieve hydrocele patients from pain. Considering that the disease falls in the group of neglected tropical disease affecting the poor of the poorest, hydrocelectomy procedures are not always accessible to majority of the patients because of the high cost of treatment as it was reported by some of the patients during samples collection in this study. Many of endemic countries have not initiated morbidity management and disability prevention programmes to take care of the affected persons.

Early diagnosis and prompt treatment of lymphatic filariasis are required for GPELF control programs to realize its goals. Access to appropriate diagnostic tools is crucial for disease control and elimination programmes in achieving their goals. Diagnostic tools are important for initial disease mapping, monitoring program progress, for deciding on when to stop the interventions and for perform post-elimination surveillances (Solomon *et al*., 2012). Use of accurate and sensitive diagnostic tools also aid in early detection of drug resistance emerging in parasite populations as a result of increased exposure to drugs due to scaling-up of MDA both in human and vectors. There is also need to track any genetic changes or variations within the parasites to improve efficiency of drugs, vaccines and diagnostic tools. Screening of large numbers of human populations and vectors is necessary when the infection levels are low. Reliability of diagnostic tools under such scenario is

vital when the decision to stop MDA in an area has to be made, as well as in the post-MDA monitoring stages. There are instances of infection resurge if low levels of the infections are overlooked even after 5-6 years of treatments as observed in studies by Minetti and colleagues, (2019) in Ghana.

In this study, (i) *W. bancrofti* 18S rRNA gene was characterized and phylogenetically analysed for isothermal molecular assays development. Sequencing and analysis of 18S rRNA gene in 14 samples from Mpirani in Malindi and Kipini in Tana River Delta was done. (ii) Two isothermal molecular assays; LAMP and HDA were developed and optimized to assess their applicability in a field set up and compared them with PCR and FTS tests.

Different amplicon detection methods were also tested; use of different intercalating dyes, turbidity observation by naked eyes, lyophilized LAMP closed tubes and agarose gel electrophoresis was used in confirmations of the results. This was aimed to obtain a simple, cheap and accurate diagnostic tools which can detect parasites in low levels as recommended by WHO for accessing progress of the control programs in any endemic areas. (iii) LAMP assay in combination with 1:10 SYBR Green 1 dye was validated in a field step up and compared to PCR for patients' blood and mosquito vectors. Antigen detection in patients was also done by FTS methods in a field set up. During validations, two regions were considered; a known-endemic region of Tana River Delta and a presumed endemic region in Matayos Busia County. Mosquito infection and infectivity rates with microfilaria larvae were also determined during assays field validation. LAMP assay was found to have high sensitivity and specificity close to that of PCR assay and with easy amplicon detection methods. Infection prevalence in the study sites was determined and descriptive statistics generated for participants and mosquito vectors.

In this study, *W. bancrofti* DNA amplification and sequencing analysis of the Ssp 1 DNA repeat sequence revealed that the region is highly conserved with some some small degree of genetic variations in *W. bancrofti* found in the two geographical areas studied in Kenyan (Mutanu *et al*., 2020). Malindi population had a divergence of 0.95 which was higher compared to Tana River population which had a divergency of 0.35. The divergence different is attributed to the long period malindi had been under MDA programmes compared to Tana River. With the recent introduction of three drug regime in Kenya (Njomo *et al.,* 2020), this initial genetic data is necessary for tracking any variations occurring in Kenyan strains. More studies are needed to track any changes that may be associated with drug pressure as noticed by many other studies in several endemic countries (De Souza *et al*., 2014; Hoti *et al.,* 2008; Wolstenholmen, 2004).

In this study, phylogenetic tree construction revealed that Kenyan isolates from both Tana River Delta and Malindi were closely related to *W. bancrofti* isolates W. b 1-07 18s rRNA accession number EU272178.1 from Brazil (Chapter 4, figure 4.5).

A total of 125 field collected samples were used for designing and optimization of LAMP and HDA assays. Statistically, LAMP assays were more accurate as compared to HDA assays using PCR as the gold standard for both methods. Comparing LAMP and HDA to PCR as the gold standard, LAMP had a higher sensitivity and specificity. There was also a higher agreement of LAMP and PCR with Kappa statistics of (K_{Value} 0.84) as compared to that of HDA which had a Lower K_{value} 0.67 (Table 5.4). This indicated that LAMP assays had closely comparable sensitivity and specificity to that of PCR and can be used to substitute PCR in resource limiting regions. By comparing the detection methods, amplicons by LAMP were easily monitored by naked eyes through the formation of white precipitation due to accumulation of magnesium pyrophosphate as a by-product, ($Mg_2P_2O_7$). The results of this study were similar to ealier observations of studies by Sakai

et al., (2017); Mashooq *et al.,* (2016) and Kumvongpin *et al.,* (2016). Florescent SYBR Green 1 dye show colour change that was observable by naked eyes, under sunshine or under a UV-light as reported in the studies by Monazah and colleagues (2017) and Singh and colleagues (2017). Use of closed tube with lyophilized reagents was found to be more suitable compared to the other amplification and detection methods but it was limited by high cost of the tubes. LAMP assays analytical sensitivity and specificity were comparable to those of PCR with Ssp I repeat target. LAMP assay could detect 1/1000000 (10^{-6}) equivalent to DNA amount of one microfilaria while HDA had a detection limit of 1/1000 (10^{-3}). Thus, by this analysis, LAMP assay was picked as the Isothermal method of choice to proceed to field validations.

During the field assay validations, participants in this study exhibited various lymphatic filariasis symptoms including, lymphedemas of one or both legs, hydrocele, leg pains as described in other studies by Dietrich and colleagues, (2019). Majority of the asymptomatic participants were positive for circulating filarial antigens or for microfilariae DNA. This is because some carriers show no signs for a period of time before the observable clinical signs manifest and these are among the risky group transmitting filariasis. Some of the patients with chronic clinical signs turned negative for CFA and for DNA detection. This could be due to the fact that the worms might have been eliminated or died long ago and are not producing microfilariae despite having caused the long-term effect. Despite the fact that Busia has not been included in the list of endemic regions in Kenya, this study found alarming chronic cases which are of a major public health concern and there is need to consider this region for lymphatic filariasis MDA control programmes.

In this study, increased prevalence was noted, this could have been brought about by new infections due to movement of people or noncompliance to MDA. It was noted that majority of people in the area were not adhering to MDA regulations even with provisions

of the drugs by the National control programs. Some patients mentioned that with the recent introduction of three drug regime, they found the drugs uncomfortable, with some reporting side effects like nausea, lack of appetite and vomiting and thus others avoided taking the drugs leading to low MDA coverage. The low coverage might adversely affect the progress of the control programmes negatively leading to increased prevalence. In Busia, the prevalence's were 17.6% by FTS and 7.3% by both PCR and LAMP. There was no previous data for lymphatic filariasis infections in this region, thus this study was the first one to be reported in this region, though the history of infections and duration confirmed that the disease has been circulating in this population.

Molecular xenomonitoring is a useful tool for evaluating the impact of lymphatic filariasis interventions on infectious microfilaria reservoir in human host. During field validations in this study, mosquito vectors of genera considered to be of medical importance for lymphatic filariasis transmissions were collected and dissected in search of microfilariae. None of the mosquitoes from Matayos had microfilaria larvae but 2 mosquitoes of *Culex* species from Tana River had microfilariae. On performing PCR and LAMP assays, two pools of *A*nopheles mosquitoes from Matayos and one pool of Culex species from Tana River were found to contain *W. bancrofti* DNA.

Detection of microfilaria in mosquitoes indicate existence of a reservoir in human host while the presence of infective third-stage larva signifies transmission potential. Therefore, xenomonitoring is an important tool in final stages of elimination program and for post-MDA evaluations as it gives the estimation of disease burden and transmission (Okorie and de Souza, 2016). According to this study, mosquitoes collected from Tana River had an infection rate of 1% and infectivity rate of 0.49%. Infections rate reduction in vectors shows valuable interruptions of transmission due to control interventions. This study also found that, there were intensive use of long-lasting mosquito nets, an intergrated vector

approach for Malaria and filariasis in both study sites. This has led to less vector-human interaction resulting to low transmission rates. Similar observations were made by Njomo and colleagues, (2020) in Kilifi and Kwale Counties and Githinji and colleagues, (2019) in Teso and West Pokot for filarial and malaria infections reduction respectively.

Detection of filarial DNA in mosquitos' pools is relatively faster and less labour intensive hence large numbers of mosquitoes are screened as compared to traditional dissection method. The method is also more sensitive and allows species-specific identification of filarial worms (Okorie and de Souza, 2016). This study focused on developing a LAMP based method for filarial DNA in pools of mosquito vectors. LAMP assay evaluated here is suitable for diagnosis of *W. bancrofti* in blood and in mosquito pools of 2 to 20 mosquitoes of the same genera per pool. The developed LAMP assay in this study can detect DNA at a lower detection limit of 10^{-6} lower than what was reported by Takagi which had a detection limit of 10^{-4}. LAMP assays have significant advantages over PCR and HDA methods. LAMP reaction uses Bst polymerase enzyme which is more torerable to inhibitors as compared to Taq polymerase used in PCR (Kaneko *et al*., 2007; Nkouawa *et al.,* 2010). Secondly, LAMP assay amplification takes 30 minutes which is a shorter time compared to PCR and HDA reactions and the results can be observed in colorimetric readout without the need for sophisticated instruments.

7.2: Conclusion

Wuchereria bancrofti 18S rRNA gene is highly conserved with minimal intraspecific variations and hence appropriate for diagnostics target for *W. bancrofti* in Kenyan endemic regions. Loop-mediated isothermal amplification assay can comfortably replace PCR method for detecting *W. bancrofti* infections in human blood and vectors. The assay is highly sensitive and species specific and matched PCR with great Kappa statistics agreement on clinical samples. LAMP is suitable for field set-ups as it uses constant

temperature that can be run on a heated block. The method offers operational simplicity, rapidity and versatility of visual readout options eliminating use of thermo-cyclers and gel electrophoresis apparatus. This diagnostic tool is of great benefit to global health programs in assessing the programmes achievements towards their goals to eliminate filariasis as a public health problem.

From epidemiological surveillance during LAMP assay validations, this study found an increasing prevalence and presence of suitable vector mosquitoes. This is an indication of on-going transmissions in Tana River and Busia Counties, thus justifying additional MDA rounds. This data therefore, allow the control programmes focus on current available resources in areas with empirical evidence of lymphatic filariasis infections. High antigenaemia and DNA detection results in Busia suggest that there are filarial infections circulating in the study population.

7.3: Recommendations

1. This study recommends for more genetic studies targeting *W. bancrofti* 18S rRNA gene and other actin gene from other endemic regions in Kenya for diagnostic targets and for proper management of the disease.
2. This study recommends the uptake of LAMP method for field surveillance and control programme to monitor progress. Use of crude specimens need also to be assessed so as to reduce time and resources for sample processing.
3. Consindering the lack of enough epidemiological data on status of infections in Busia County and other susceptible regions, this study recommends re-mapping of lymphatic filariasis in Kenya.
4. I call upon the county governments of these regions to take initiative in providing diagnostic tools in health centers and referral hospitals for early diagnosis and prompt treatment to curb disability caused by filarial infections.

5. This study also recommends the National control programmes to initiate home based care for morbidity management and disability prevention since majority of the victims found had chronic stages of the disease and were suffering silently.

8.0: STUDY LIMITATIONS

1. This study used few specimens for genetic characterization and from only 2 endemic regions thus difficult to draw conclusion on genetic diversity, neutrality and selection.
2. The cost of optimizing the molecular assays were tool high and there was a lot of time spent to completely optimize and validate the assays.
3. There was a lot of insecurity issues during samples collection in Tana River Delta.

9.0: REFERENCES

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10.0: APPENDICES

Appendix I: Research approval for Tana River County study sites

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 19, 2014**

TO: **NANCY KINYATTA**
PRINCIPAL INVESTIGATOR

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

25/8/14

Dear Madam,

RE: **SSC PROTOCOL NO. 2802 *(RESUBMISSION):* EVALUATION AND APPLICATION OF ISOTHERMAL AMPLIFICATION METHODS AND OLIGOCHROMATOGRAPHIC DIPSTICK AS POINT-OF-CARE-TOOLS FOR THE DIAGNOSIS OF *WUCHERERIA BANCROFTI* IN HUMAN BLOOD AND MOSQUITO VECTORS (*VERSION 3. DATED 12 /08/2014*)**

Reference is made to your letter dated August 12, 2014. The ERC Secretariat acknowledges receipt of the revised protocol on 15th August, 2014.

This is to inform you that the Ethics Review Committee (ERC) reviewed the documents submitted 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 **19th August, 2014**. Please note that authorization to conduct this study will automatically expire on **August 18, 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 7, 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.

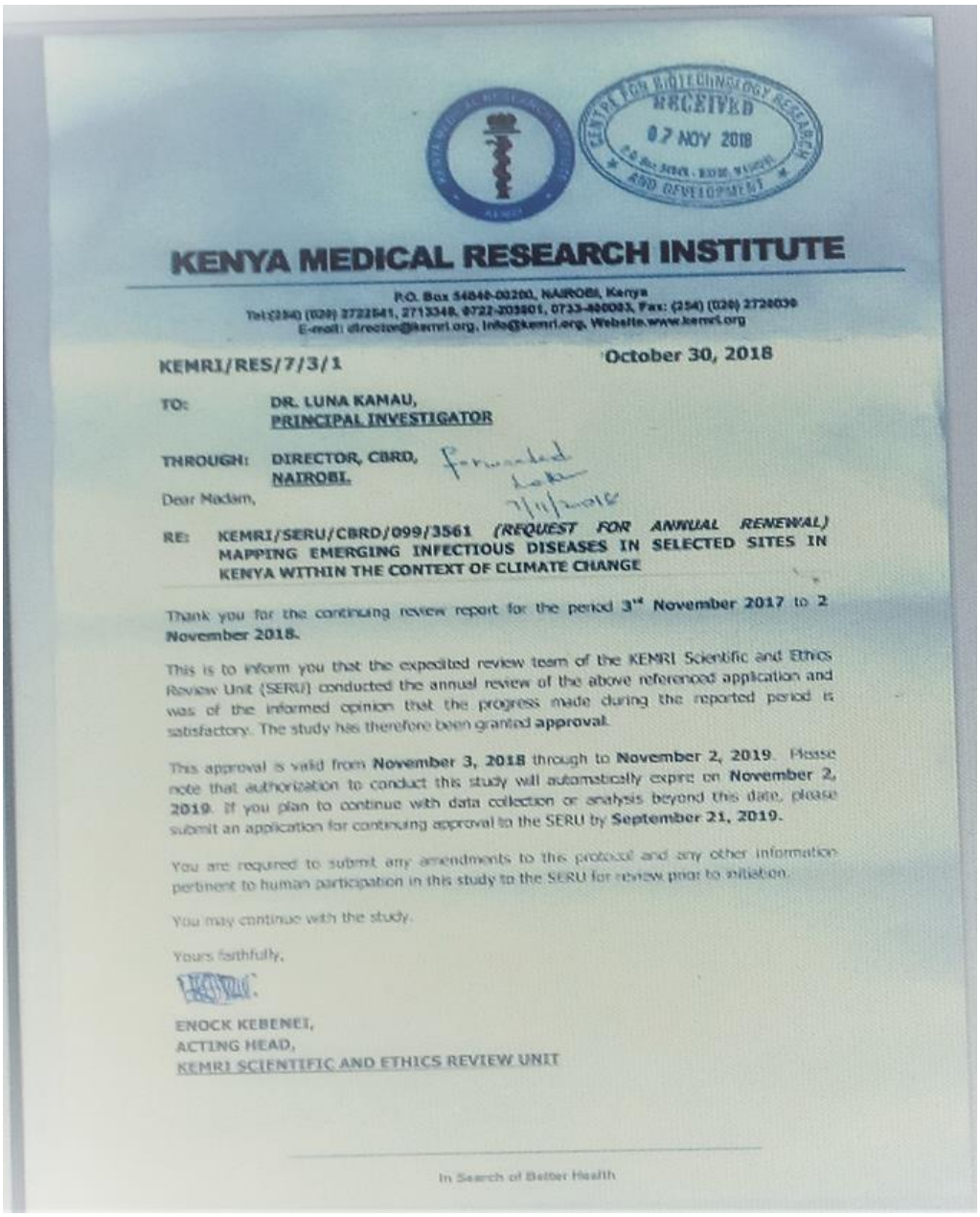
Yours faithfully,

EAB

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

In Search of Better Health

Appendix II: Research approval for Matayos Busia County study site:



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KENYA MEDICAL RESEARCH INSTITUTE

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KEMRI/RES/7/3/1 October 30, 2018

TO: DR. LUNA KANAU,
PRINCIPAL INVESTIGATOR

THROUGH: DIRECTOR, CBRD,
NAIROBI.

Forwarded
7/11/2018

Dear Madam,

RE: KEMRI/SERU/CBRD/099/3561 *(REQUEST FOR ANNUAL RENEWAL)*
MAPPING EMERGING INFECTIOUS DISEASES IN SELECTED SITES IN KENYA WITHIN THE CONTEXT OF CLIMATE CHANGE

Thank you for the continuing review report for the period 3rd November 2017 to 2 November 2018.

This is to inform you that the expedited review team of the KEMRI Scientific and Ethics Review Unit (SERU) conducted the annual review of the above referenced application and was of the informed opinion that the progress made during the reported period is satisfactory. The study has therefore been granted approval.

This approval is valid from November 3, 2018 through to November 2, 2019. Please note that authorization to conduct this study will automatically expire on November 2, 2019. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the SERU by September 21, 2019.

You are required to submit any amendments to this protocol and any other information pertinent to human participation in this study to the SERU for review prior to initiation.

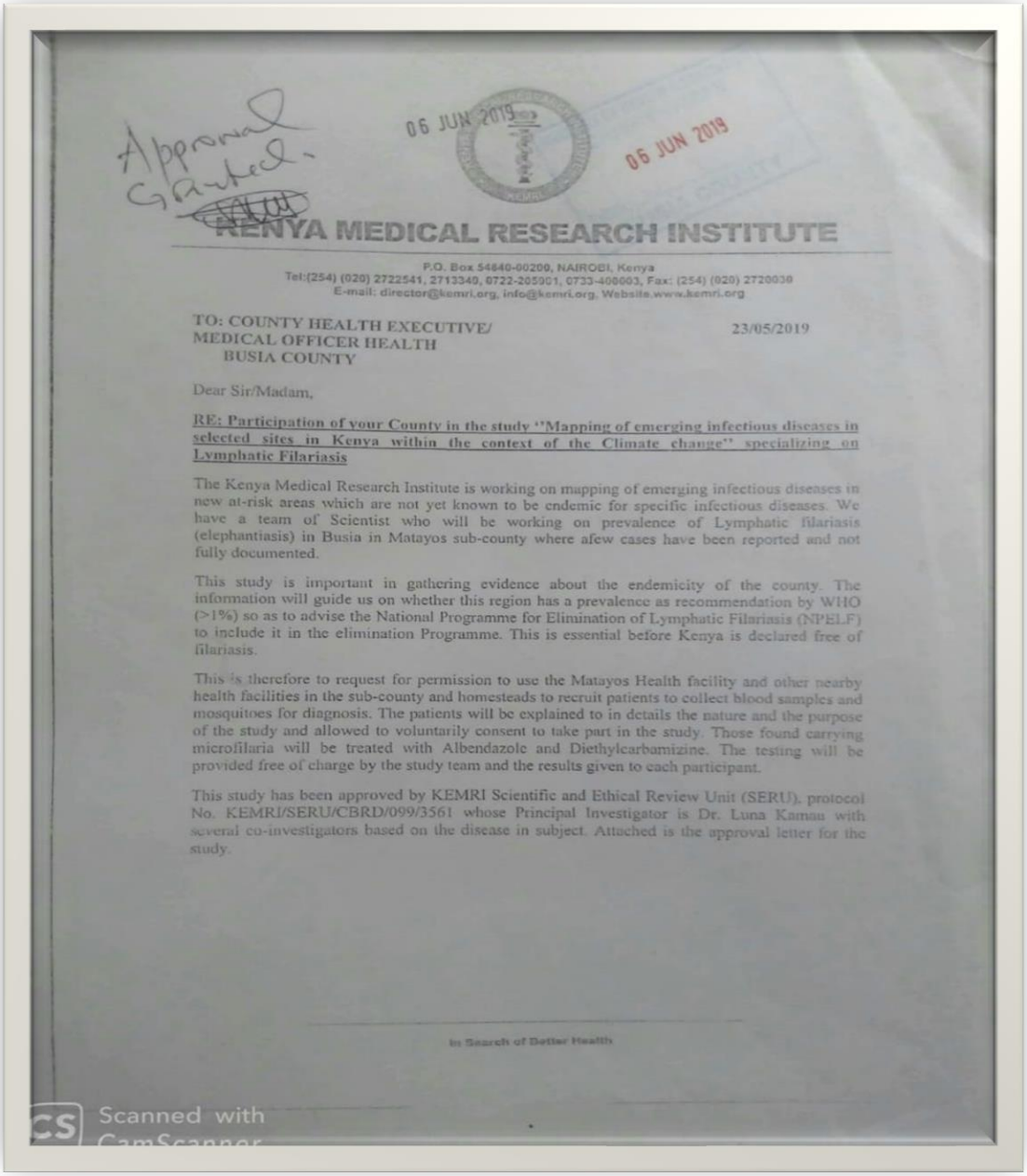
You may continue with the study.

Yours faithfully,

ENOCK KEBENEI,
ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

Appendix III: Busia County study approval letter



Approval Granted

06 JUN 2019

06 JUN 2019

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel:(254) (020) 2722541, 2713340, 0722-205901, 0733-400603, Fax: (254) (020) 2720030
E-mail: director@kemri.org, info@kemri.org, Website.www.kemri.org

TO: COUNTY HEALTH EXECUTIVE/
MEDICAL OFFICER HEALTH
BUSIA COUNTY

23/05/2019

Dear Sir/Madam,

RE: Participation of your County in the study "Mapping of emerging infectious diseases in selected sites in Kenya within the context of the Climate change" specializing on Lymphatic Filariasis

The Kenya Medical Research Institute is working on mapping of emerging infectious diseases in new at-risk areas which are not yet known to be endemic for specific infectious diseases. We have a team of Scientist who will be working on prevalence of Lymphatic filariasis (elephantiasis) in Busia in Matayos sub-county where afew cases have been reported and not fully documented.

This study is important in gathering evidence about the endemicity of the county. The information will guide us on whether this region has a prevalence as recommendation by WHO (>1%) so as to advise the National Programme for Elimination of Lymphatic Filariasis (NPELF) to include it in the elimination Programme. This is essential before Kenya is declared free of filariasis.

This is therefore to request for permission to use the Matayos Health facility and other nearby health facilities in the sub-county and homesteads to recruit patients to collect blood samples and mosquitoes for diagnosis. The patients will be explained to in details the nature and the purpose of the study and allowed to voluntarily consent to take part in the study. Those found carrying microfilaria will be treated with Albendazole and Diethylcarbamizine. The testing will be provided free of charge by the study team and the results given to each participant.

This study has been approved by KEMRI Scientific and Ethical Review Unit (SERU), protocol No. KEMRI/SERU/CBRD/099/3561 whose Principal Investigator is Dr. Luna Kamau with several co-investigators based on the disease in subject. Attached is the approval letter for the study.

In Search of Better Health

Appendix IV: Informed consent document (ICD) I: Mapping emerging infectious diseases in Kenya within the context of climate change

Principal Investigators; Luna Kamau

Co-Principal Investigators: Nancy Kinyatta, Anthony Muthee, David Muriu, Josyline Cirindi, Benard Osero, and Francis Kimani

Co-Investigators: Rosemary Githae, Jim Kagai, Jacinta Muli, Milka Mwangi, Christopher Anjili, Ruttoh Reuben, Damaris Matoke, Linus M'rewa, Rael Musili, Joseph Mwatha and Edwin Too

Institutional affiliation:

[1] Centre for Biotechnology Research and Development, Kenya Medical Research Institute, P.O Box 54840-0020

1. Introduction and participant information

Kenya Medical Research Institute, conduct research with a mission of improving human health, capacity building and service delivery towards achieving the Vision 2030 and the Millennium Development Goals. KEMRI will conduct a study to investigate the presence of *Wuchereria bancrofti* causing elephantiasis in human.

The aim is to investigate whether there is transmission of the parasite in mosquitoes and human blood in the population as evidenced by the lymphedemas experienced in the study area. The aim of this study is to to establish the cause of the lymphedemas in this region for lymphatic filariasis to advices the policy makers and Ministry of Healthy for appropriate control measures. We are requesting you to take part in this study. We will need you to have finger prick for blood collection and if found to have circulating filarial antigens we will request you for 2ml veinous blood. Please take time to read this explanation to the study, and when you have read, feel free to ask questions or to seek clarification on any issues related to this study or your participation in it.

2. Purpose of the study.

To investigate the presence of *Wuchereria bancrofti* parasite causing elephantiasis in humans and that there is evidence of transmission in mosquitoes and human blood in the population as evidenced by the lymphedemas experienced in the study area.

3. Procedures

As a participant, you are requested to consent for finger prick blood collection and if found to be positive for circulating filarial antigens you will also be requested to give venous blood.

4. Maintenance of confidentiality

You will be assigned a coded number, and you or results of tests done on samples taken from you will be referred to by this number in all correspondence or publications arising from this study.

5. Benefits

There will be no MDA done in this study, however the participants found to be having filarial parasites will be treated with a combined dose Albendazole and Diethylcarbamizine. The community will be educated on the control measures of the disease.

6. Risks

The risks involved here are minimal. There will be minimal acceptable pain caused by needle pricks during blood collection.

7. **Contact of KEMRI Scientific Ethical Review Unit**

P.O box 54840, Tel, 2722541 (Weekdays, daytime).

8. Compensation

No compensation involved.

9. Sample storage, exportation and analysis

ICT test will be carried out in the study area and results recorded and given to individual participants. The cards will be carried back to KEMRI-NAIROBI to be used in case of further clarification in recording of results for analysis. Blood samples will be packed well in cooler boxes with ice packs and transported to KEMRI Radio Isotope Laboratory in Nairobi where DNA extraction and amplification will take place.

10. Consent and signature options

Participant's Name----------

Date of Birth: ----------Age----------Sex----------

Address: ----------

PARTICIPANT'S SIGNATURE ……

Assent for children below 18years and challenged persons

Title: Mapping emerging infectious diseases in Kenya within the context of climate change

Principal Investigators; Luna Kamau

Co-Principal Investigators: Anthony Muthee, David Muriu, Josyline Cirindi, Benard Osero, Nancy Kinyatta and Francis Kimani

Co-Investigators: Rosemary Githae, Jim Kagai, Jacinta Muli, Milka Mwangi, Christopher Anjili , Ruttoh Reuben, Damaris Matoke, Linus M'rewa, Rael Musili, Joseph Mwatha and Edwin Too.

Institutional affiliation:

[1] Centre for Biotechnology Research and Development, Kenya Medical Research Institute, P.O Box 54840-0020

Participant's Name----------

Date of birth: ----------Age ----------Sex----------

Assent: ----------

I, Parent/guardian have full capacity to assent on behalf of ----------

Parents/guardian's signature----------

Kiswahili version

Andiko la makubaliano ya kujulisha

Uchunguzi wa kuthibitisha uwepo wa wadudu wanaosababisha ugonjwa wa Matende (Jethamu) kutumia damu ya binadamu na mbu unaoeneza.

1. Utangulizi na habari za Mhusika

Kituo cha utafiti wa kisayansi cha KEMRI ufanya utafiti na kusudi la kuendeleza Afya ya binadamu na udhamana wa maisha, kujenga uwezo na pia jinsi ya kutoa huduma ili kufikia maono ya 2030, na malengo ya maendeleo ya karne hii. KEMRI itafanya uchunguzi wa kuthibitisha uwepo wa wadudu wanaosababisha ugonjwa wa Matende (Jethamu) kutumia damu ya binadamu na mbu unaoeneza. Hakuna kusudi la kujipatia pesa katika somo hili lakini kusudi ni kujua kama kuna uenezaji wa matende katika sehemu hii ili kujua njia mwafaka itakayo tumika kwa kuthibiti hii ungonjwa. Kama watu wanao husishwa katika somo hili, tunawauliza muhusike katika somo. Kuhusika ni kwa hiari, uko na haki ya

kukataa au kujusuru bila kupoteza faida yeyote. Tafadhali chukua muda kusoma hamasisho hili la somo. Utakapo maliza kusoma huko huru kuuliza maswali ama maelezo kuhusu jambo lolota linalohusu somo hili. Utaulizwa kutolewa damu itakayotumika kwa utafiti huu.

2. Kusudi la somo

Kusudi la somo hili ni kujua kama kuna uenezaji wa ugonjwa wa matende katika sehemu hili ili kujua njia mwafaka itakayo tumika kwa kuthibiti ungonjwa huu wa matende kwa binadamu na mbu wanaoeneza huu ugonjwa.

3. Utaratibu

Mhusika ataombwa kukubali kutolewa damu kwa kidole na pia kwa mshipa kama atapatikana na wadudu kwa damu.

4. Kuifadhi siri

Nambari ya kitambulisho chako na matokeo ya uchunguzi yatabaki siri. Kama mhusika utapewa taraimu (nambali) ya siri itakayotumiwa badala ya Majina yako. Hivyo basi wewe au sampuli ya uchunguzu wako itatambulika kwa tarakimu katika mawasiliano ya waraka au uandishi utokanao na somo hili habari zote za uchunguzi wa kimatibabu zitabaki siri na sitabaki na mdadisi mkuu wa somo.

5. Faida

Kwa watakao patikana na maradhi ya matende watapewa matibabu ya dawa za minyoo(Albendazole na Diethylcarbamizine). Watu watafunzwa njia mwafaka za kujikinga na ugonjwa huu. Matokeo yatakua ya faida kwa miradi inayohusika kwa kuthibidi ugonjwa huu na wenyeji watafaidika na dawa za kuzuhia ugonjwa wa matende zinazopeanwa na washika dao wanaofaa.

6. Hatari

Hatari zilizomo ni kidogo sana, kuna uchungu kidogo unao sababishwa na sindano wakati wa kutolewa damu ya sampuli. Tahadhali itatiliwa maanani wakati wa kutolewa damu na wauguzi wenye kuitimu ndio watakao fanya kazi hiyo.

7. Maswali ya KEMRI/kamati ya taifa ya marejeleo ya maadili

Kama unaitaji kuuliza kuhusu usalama na haki hili kuhusika katika somo hili kituo kinacho husika ni KEMRI SLP 54840 Simu 2722541.

8. Fidhia

Hakutakua na kupoteza au hatari yeyote kubwa inayohusu somo hili, hivo basi hakuna fidhia yeyote inatarajiwa.

9. Jinsi ya kuifadhi na kusafirisha sampuli kwa uchungusi zaidi

Jaribio la kitekinologia la tarakilishi litafanywa katika eneo la somo na matokeo yatanakiliwa na kupatiwa mhusika. Kadi zitabebwa na kupelekwa KEMRI Nairobi ili kutumiwa kama kutakuwa na maelezo ya wazi zaidi ya rekodi ya matokeo, ili kudadisi zaidi.

Vichupa vya sampuli vitawekwa katika visanduku vyenye barafu na kusafirishwa hadi KEMRI katika mahabara ya miale ya atomiki na nukilia Nairobi ambapo *W. bancrofti* DNA sitatenganishwa.

10. Chaguo la makubaliano na sahii

Jina la mhusika----------

Tareha ya kusaliwa----------miaka----------umbo

S.L.P----------

Mimi---------- nina uwezo wa makubaliano. Nimejulishwa kuhusu somo kwa makini. Nimesoma habari niliyohelesewa na nikaelewa. Nimekubali kutolewa damu kwa hiari pia mbu kuokotwa kwa nyumba yangu. Pia ninaelewa kwamba naweza kujuusuru kutokana na somo.

Sahii ya mhusika ama alama ya kidoe gumba cha kushoto ----------

11. Makubaliano ya watoto chini ya miaka kumi na nane na walemavu

Jina la mhusika----------

Tarehe ya kusaliwa----------miaka----------umbo

SLP----------

Mimi---------- (Mzazi) kwa niaba ya---------- (Mtoto)

Nimejulishwa kuhusu somo kwa makini. Nimesoma habari niliyohelesewa na nikaelewa. Nimekubali atolewa damu kwa hiari na Pia ninaelewa kwamba anaweza kujusuru kutokana na somo.

Appendix V; Informed consent document (ICD) II: Evaluation of isothermal amplification methods and development of an OligoChromatographic dipstick assay for point-of-care diagnosis of *Wuchereria bancrofti* and disease surveillance

Principal investigator: Nancy Kinyatta [1]

Co- Investigators: Jim Kagai[1], Luna Kamau[1], Lillian Wambua[2, 3], Claire M. Mugasa[4], Francis Kimani[1], Rosemary Githae[1] and Dorcas Wachira[1]

Institutional affiliation:

[1] Centre for Biotechnology Research and Development, Kenya Medical Research Institute, P.O Box 54840-0020

[2]University of Nairobi, School of Biological Science, P.O. Box 30197-00100

[3]International Centre for Insect Physiology and Ecology. P.O Box 30772-00100, Nairobi, Kenya

[4]Dept of Biotechnical and Diagnostic sciences, College of Veterinary Medicine, Animal resources and Biosecurity (COVAB), Makerere University, P. O. Box 7062 Kampala, Uganda

1. Introduction and study details

The Kenya Medical Research Institute (KEMRI), conduct research with the mission of improving human health, capacity building and service delivery towards achieving the Vision 2030 and the Millennium Development Goals. KEMRI will conduct a study to look for easy and cheap methods of *Wuchereria bancrofti* diagnosis where the affected people are found (point of care).

The aim is to establish a diagnostic method which is an effective point of care diagnostic tool applicable in resource-poor endemic areas without requiring a lot of expertise and sophisticated equipments. The main goal is to get a simpler method of diagnosing lymphatic filariasis that can be used in monitoring control programs and also in mapping areas where MDA is required at a reduced cost to aid GPELF. The tools will also be used to determine when to stop giving the drugs when the disease transmission is reduced to levels which are not of much public health concern. The participation is voluntary.

2. Purpose of the study.

To look for new ways to use in testing elephantiasis (*Wuchereria bancrofti* diseases)

3. Procedures

You are requested to consent for 4ml of venous blood collection and allow for mosquitoes to be sampled from your houses.

4. Maintenance of confidentiality

You will be assigned a number, and you or results of tests done on samples taken from you will be referred to by this number in all correspondence or publications arising from this study. All information and medical records will remain confidential and will only be accessible to the investigator in this study.

5. Benefits

There will be no mass drug administration done in this study, however the participants found to be having filarial parasites will be treated with a combined dose of Albendazole and Diethylcarbamizine. You will be educated on the control measures of the disease. There will be no losses or major risks involved in this study so no compensation should be expected.

6. Risks

Only minimal pain involved.

7. Sample storage, exportation and analysis

Blood samples will be packed well in cooler boxes with ice packs and transported to KEMRI Radio Isotope Laboratory in Nairobi where DNA extraction and amplification will take place.

8 Contact of Principal Investigator

This study is under the direction of NANCY M. KINYATTA ID no 22090982 and in case of any problem contact me at KEMRI, box 54840, Tel, 2722541.

9 Contact of KEMRI Ethical Review Committee

In case you need to enquire about your security and right to participate in this study, you will contact; The Secretary, KEMRI Ethical Review Committee P.O box 54840-00200, Telephone numbers 020-2722541, Mobile **+254717719477,** 0722205901, 0733400003; email address: ERCadmin@kemri.org. Contact Time**: 8:00 AM to 5:00 PM within week days.**

10 Consent and signature options

Participant's Name----------

Date of birth: ----------Age----------Sex----------

Address: ----------

I---------- have full capacity to consent. I allow the researchers to withdraw blood and collect mosquitoes from my house.

PARTICIPANT'S SIGNATURE …….

Assent for children 12-17 years old

Title: Evaluation of isothermal amplification methods and development of an OligoChromatographic dipstick assay for point-of-care diagnosis of *Wuchereria bancrofti* and disease surveillance

Principal investigator: Nancy Kinyatta [1]

Co- Investigators: Jim Kagai[1], Luna Kamau[1], Lillian Wambua[2, 3], Claire M. Mugasa[4], Francis Kimani[1], Rosemary Githae[1] and Dorcas Wachira[1]

Institutional affiliation:

[1] Kenya Medical Research Institute, Centre for biotechnology Research and development, P.O Box 54840-0020

[2]University of Nairobi, School of Biological Science, P.O. Box 30197-00100

[3]International Centre for Insect Physiology and Ecology, P.O Box 30772-00100, Nairobi, Kenya

[4]Dept of Biotechnical and Diagnostic sciences, College of Veterinary Medicine, Animal resources and Biosecurity (COVAB), Makerere University, P. O. Box 7062 Kampala, Uganda

How are you? My name is Nancy Kinyatta. My colleagues and I are research scientists from KEMRI. We would like to talk to you about a research study that we would like to carry out. To begin with a research study is a scientific experiment that is carried out by a scientist or doctor to solve or answered some mysteries. It is carried out to investigate or gather information about something. In our research study we like to evaluate some methods which can be used to test elephantiasis disease in a field set up. After explaining to you on how the study will be conducted.

If you agree to participate in the study, you will provide us with a small amount of blood and allow us to collect mosquitoes in your houses. We will not take much of your time for blood collection will only take about five minutes and you will be allowed to leave for your other errands.

Confidentiality; You will be assigned a coded number. Your personal information will never be made public to other researchers or anyone else. Only the coded numbers will be used during sample analysis and data management.

Benefits; You will be treated with a dose of Albendazole and Diethylcarbamizine.

Compensation; No compensation will be offered.

Storage, Transportation of samples; the samples will be transported to KEMRI- Radio Isotope laboratory where the analysis will take place.

Agreement 12-17 years old

Title: Evaluation of isothermal amplification methods and development of an OligoChromatographic dipstick assay for point-of-care diagnosis of *Wuchereria bancrofti* and disease surveillance

Principal investigator: Nancy Kinyatta [1]

Co- Investigators: Jim Kagai[1], Luna Kamau[1], Lillian Wambua[2, 3], Claire M. Mugasa[4], Francis Kimani[1], Rosemary Githae[1] and Dorcas Wachira[1]

Institutional affiliation:

[1] Centre for Biotechnology Research and Development, Kenya Medical Research Institute, P.O Box 54840-0020

[2]University of Nairobi, School of Biological Science, P.O. Box 30197-00100

[3]International Centre for Insect Physiology and Ecology. P.O Box 30772-00100, Nairobi, Kenya

[4]Dept of Biotechnical and Diagnostic sciences, College of Veterinary Medicine, Animal resources and Biosecurity (COVAB), Makerere University, P. O. Box 7062 Kampala, Uganda

I---------- wish to participate in this research being conducted by Nancy and colleagues of CBRD, KEMRI and P.O Box 54840 Nairobi-Kenya. I can contact Nancy. M. K at KEMRI, box 54840, Tel, 2722541 or ERC-KEMRI on Email ERCAdmin@kemri.org.

Participants Name----------

Parents/guardian Name----------

Date of Birth----------Age ----------Sex----------

Address---------- Signature----------

12.0 Assent for children below 12 years and challenged persons

Title: Evaluation of isothermal amplification methods and development of an OligoChromatographic dipstick assay for point-of-care diagnosis of *Wuchereria bancrofti* and disease surveillance

Principal investigator: Nancy Kinyatta [1]

Co- Investigators: Jim Kagai[1], Luna Kamau[1], Lillian Wambua[2, 3], Claire M. Mugasa[4], Francis Kimani[1], Rosemary Githae[1] and Dorcas Wachira[1]

Institutional affiliation:

[1] Centre for Biotechnology Research and Development, Kenya Medical Research Institute, P.O Box 54840-0020

[2]University of Nairobi, School of Biological Science, P.O. Box 30197-00100

[3]International Centre for Insect Physiology and Ecology. P.O Box 30772-00100, Nairobi, Kenya

[4]Dept of Biotechnical and Diagnostic sciences, College of Veterinary Medicine, Animal resources and Biosecurity (COVAB), Makerere University, P. O. Box 7062 Kampala, Uganda

Subject's Name----------

Date of birth: ----------Age----------Sex----------

Address: ----------

I, Parent/guardian have full capacity to assent on behalf of ----------

Parents/guardian's signature----------

Kiswahili version

Title: Evaluation of isothermal amplification methods and development of an OligoChromatographic dipstick assay for point-of-care diagnosis of *Wuchereria bancrofti* and disease surveillance

Principal investigator: Nancy Kinyatta [1]

Co- Investigators: Jim Kagai[1], Luna Kamau[1], Lillian Wambua[2, 3], Claire M. Mugasa[4]**,** Francis Kimani[1], Rosemary Githae[1] and Dorcas Wachira[1]

Institutional affiliation:

[1] Centre for Biotechnology Research and Development, Kenya Medical Research Institute, P.O Box 54840-0020

[2]University of Nairobi, School of Biological Science, P.O. Box 30197-00100

[3]International Centre for Insect Physiology and Ecology, P.O Box 30772-00100, Nairobi, Kenya

[4]Dept of Biotechnical and Diagnostic sciences, College of Veterinary Medicine, Animal resources and Biosecurity (COVAB), Makerere University, P. O. Box 7062 Kampala, Uganda.

Andiko la makubaliano ya kujulisha

Uchunguzi wa kuthibitisha utumiaji wa njia mpya mwafaka ya kupima ugonjwa wa Matende (Jethamu) kutumia ndamu ya binadamu na mbu unaoeneza.

1. Utangulizi na habari za Mhusika

Kituo cha utafiti wa kisayansi cha KEMRI ufanya utafiti na kusudi la kuendeleza Afya ya binadamu na udhamana wa maisha, kujenga uwezo na pia jinsi ya kutoa huduma ili kufikia maono ya 2030, na malengo ya maendeleo ya karne hii. KEMRI itafanya somo la njia tofauti za kupima ugonjwa wa matende. Kusudi la somo hili ni kujua Njia mwafaka zinazo tumiwa kwa uraisi na zenye zinawenza tumika hata mashinani kupima ugonjwa wa matende mbila kuitaji maharifa makubwa. Hakuna kusudi la kujipatia pesa katika somo hili lakini kusudi ni kufahamu vile hii njia mpya inaweza kutumiwa na mirandi ya kuzuia uenezaji wa matende pia kujua kiwango wameweza kuthibiti matende na hata kujua sehemu ziginezo zinazoitaji dawa za kuthibiti matende. Njia hii inaweza kutumika hata kwa kupima na kujua kiwago hii mirandi imeweza kuthimbidi au kumaliza uenezaji wa huu ugonjwa. Kama watu wanao husishwa katika somo hili, tunawauliza muhusike katika

somo. Kuhusika ni kwa hiari, uko na haki ya kukataa au kujusuru bila kupoteza faida yeyote. Tafadhali chukua muda kusoma hamasisho hili la somo. Utakapo maliza kusoma huko huru kuuliza maswali ama maelezo kuhusu jambo lolota linalohusu somo hili.

2. Kusudi la somo

Kusudi lasomo hili ni kujua njia mwafaka na ya uraisi inawenza kutumiwa kupima ugonjwa wa matende kwa binadamu na mbu wanaoeneza huu ugonjwa.

3. Utaratibu

Kama mhusika unaombwa kukubali kutolewa damu kwa mshipa na kuruhusu uokotaji wa mbu kutoka kwa nyumba yake.

4. Kuifadhi siri

Nambari ya kitambulisho chako na matokeo ya uchunguzi yatabaki siri. Kama mhusika utapewa taraimu (nambali) ya siri itakayotumiwa badala ya Majina yako. Hivyo basi wewe au sampuli ya uchunguzu wako itatambulika kwa tarakimu katika mawasiliano ya waraka au uandishi utokanao na somo hili habari zote za uchunguzi wa kimatibabu zitabaki siri na sitabaki na wadadisi mkuu wa somo.

5. Faida

Kwa watakao patikana na maradhi yo matende watapewa matibabu ya ndawa za minyoo(Albendazole na Diethylcarbamizine). Watu watafunzwa njia mwafaka za kujikinga na ugonwa huu. Matokeo yatakua ya faida kwa miradi inayohusika kwa kuthibidi ugonjwa huu na wenyeji watafaidika na dawa za kuzuhia ugonjwa wa matende zinazopeanwa na washika dao wanaofaa.

6. Hatari

Hatari zilizomo ni kidogo sana, kuna uchungu kidogo unao sababishwa na sindano wakati wa kutolewa damu ya sampuli. Tahadhali itatiliwa maanani wakati wa kutolewa damu na wauguzi wenye kuitimu ndio watakao fanya kazi hiyo. Hakutakua na kupoteza au hatari yeyote kubwa inayohusu somo hili, hivo basi hakuna fidhia yeyote inatarajiwa.

7. Jinsi ya kuifadhi na kusafirisha sampuli kwa uchungusi zaidi

Jaribio la kitekinologia la tarakilishi litafanywa katika eneo la somo na matokeo yatanakiliwa na kupatiwa mhusika. Kadi zitabebwa na kupelekwa KEMRI Nairobi ili kutumiwa kama kutakuwa na maelezo ya wazi zaidi ya rekodi ya matokeo, ili kudadisi zaidi.

Vichupa vya sampuli vitawekwa katika visanduku vyenye barafu na kusafirishwa hadi KEMRI katika mahabara ya miale ya atomiki na nukilia Nairobi ambapo *W. bancrofti* DNA sitatenganishwa.

8. Mawasiliano ya mdadisi mkuu

Somo hili liko chini ya mwongozo wa Nancy Mutanu K. numba ya kitambulisho 22090982, kama kutakua na tatizo lolote wewe mhusika unaulizwa kuwasiliana nami hapa KEMRI SLP 54840 Nairobi, simu 020 2722541

9. Maswaliano na KEMRI/kamati ya taifa ya marejeleo ya maadili

Kama unaitaji kuuliza kuhusu usalama na haki hili kuhusika katika somo hili kituo kinacho husika ni KEMRI SLP 54840 Mobile **+254717719477,** 0722205901, 0733400003; email address: ERCadmin@kemri.org. Contact Time**: 8:00 AM to 5:00 PM (GMT +3 Nairobi) week days.**

10. Chaguo la makubaliano na sahii

Jina la mhusika----------

Tareha ya kusaliwa----------miaka----------umbo

S.L.P----------

Mimi---------- nina uwezo wa makubaliano. Nimejulishwa kuhusu somo kwa makini. Nimesoma habari niliyohelesewa na nikaelewa. Nimekubali kutolewa damu kwa hiari pia mbu kuokotwa kwa nyumba yangu. Pia ninaelewa kwamba naweza kujuusuru kutokana na somo bila madhara yoote.

Sahii ya mhusika ama alama ya kidoe gumba cha kushoto ----------

11. Makubaliano ya watoto Kati ya miaka kumi na miwili na kumi na saba

Jina la mhusika----------

Tarehe ya kusaliwa----------miaka----------umbo

SLP----------

Nimejulishwa kuhusu somo kwa makini. Nimesoma habari niliyohelesewa na nikaelewa. Nimekubali kutolewa damu kwa hiari na Pia ninaelewa kwamba anaweza kujusuru kutokana na somo.

12. Makubaliano ya watoto chini ya miaka kumi na miwili na walemavu

Jina la mhusika----------

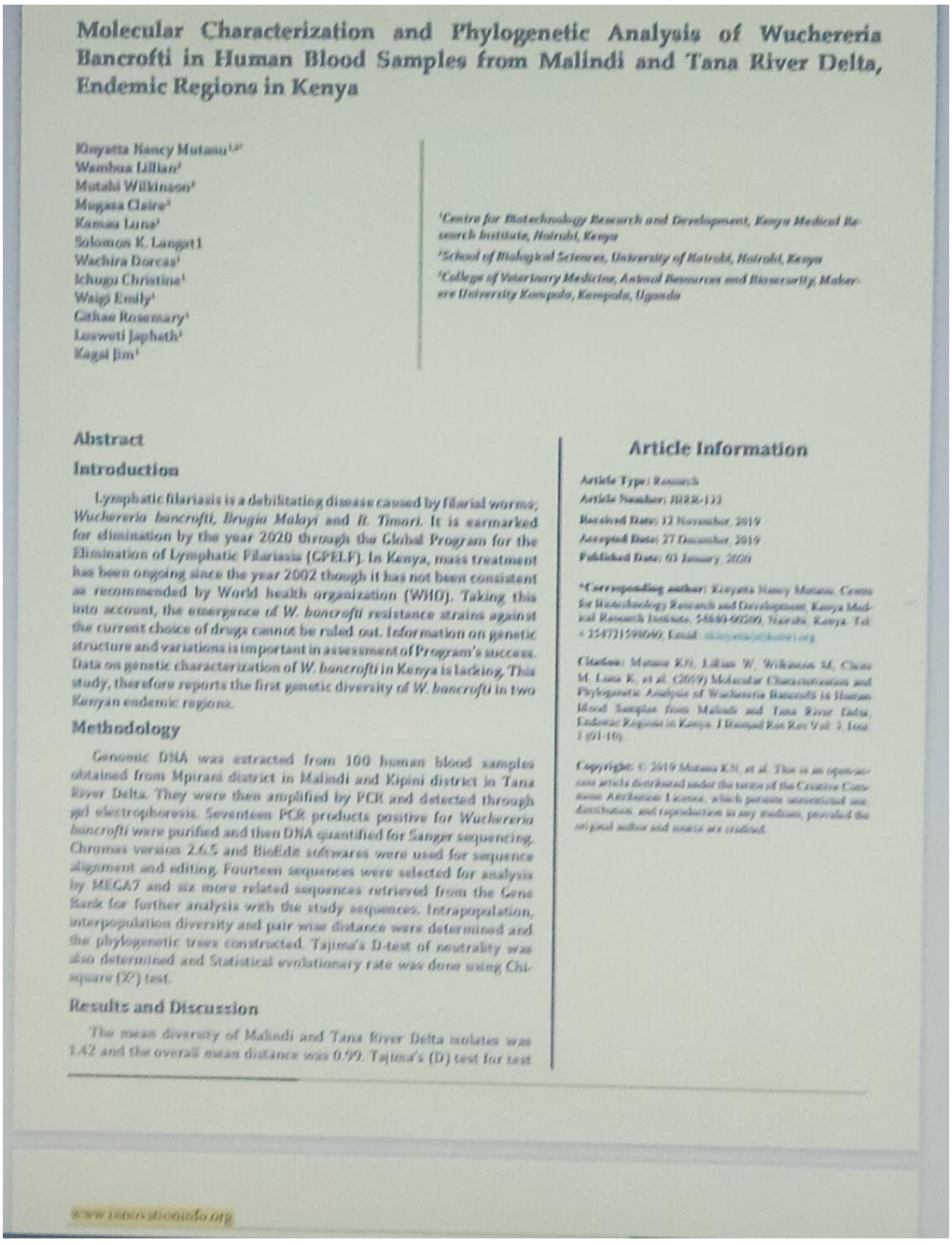
Tarehe ya kusaliwa----------miaka----------umbo

SLP----------

Mimi---------- (Mzazi) kwa niaba ya---------- (Mtoto)

Nimejulishwa kuhusu somo kwa makini. Nimesoma habari niliyohelesewa na nikaelewa. Nimekubali atolewa damu kwa hiari na Pia ninaelewa kwamba anaweza kujusuru kutokana na somo.

Appendix VI: First Publication abstract in Journal of Biomedical Research and Reviews, 2019 Volume 3: 1 ISSN: 2581-7388



Molecular Characterization and Phylogenetic Analysis of Wuchereria Bancrofti in Human Blood Samples from Malindi and Tana River Delta, Endemic Regions in Kenya

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Abstract

Introduction

Lymphatic filariasis is a debilitating disease caused by filarial worms; *Wuchereria bancrofti, Brugia Malayi* and *B. Timori*. It is earmarked for elimination by the year 2020 through the Global Program for the Elimination of Lymphatic Filariasis (GPELF). In Kenya, mass treatment has been ongoing since the year 2002 though it has not been consistent as recommended by World health organization (WHO). Taking this into account, the emergence of *W. bancrofti* resistance strains against the current choice of drugs cannot be ruled out. Information on genetic structure and variations is important in assessment of Program's success. Data on genetic characterization of *W. bancrofti* in Kenya is lacking. This study, therefore reports the first genetic diversity of *W. bancrofti* in two Kenyan endemic regions.

Methodology

Genomic DNA was extracted from 100 human blood samples obtained from Mpirani district in Malindi and Kipini district in Tana River Delta. They were then amplified by PCR and detected through gel electrophoresis. Seventeen PCR products positive for *Wuchereria bancrofti* were purified and then DNA quantified for Sanger sequencing. Chromas version 2.6.5 and BioEdit softwares were used for sequence alignment and editing. Fourteen sequences were selected for analysis by MEGA7 and six more related sequences retrieved from the Gene Bank for further analysis with the study sequences. Intrapopulation, interpopulation diversity and pair wise distance were determined and the phylogenetic trees constructed. Tajima's D-test of neutrality was also determined and Statistical evolutionary rate was done using Chi-square (X^2) test.

Results and Discussion

The mean diversity of Malindi and Tana River Delta isolates was 1.42 and the overall mean distance was 0.99. Tajima's (D) test for test

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Appendix VII Second Publication: Research Article;

Hindawi
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Hindawi

Research Article

Optimization of a Loop-Mediated Isothermal Amplification Assay as a Point-of-Care Tool for the Detection of *Wuchereria bancrofti* in Human Blood in Tana River Delta, Kenya

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Introduction. Accurate detection of filarial parasites in humans and vectors is essential for the implementation and evaluation of Global and National Programs to eliminate lymphatic filariasis. Immunological methods to detect infection are available; however, cross-reactivity issues have been reported in most of them. Nucleic acid-based molecular assays offer high levels of specificity and sensitivity and can be used to detect the infections. *Methods.* In this study, we evaluated loop-mediated isothermal amplification (LAMP) tests to amplify *Wuchereria bancrofti* DNA in patients' blood. The amplicons were tested by both pH-sensitive dyes for enhanced visual detection and agarose gel electrophoresis. A closed-tube LAMP assay was also evaluated. Cohen's Kappa statistics was used for statistical analysis of the assays. 125 patients consented for blood sampling which were used for clinical analysis of LAMP assays with the PCR method used as the "gold standard." *Results.* The sensitivity of the evaluated *Wuchereria bancrofti* LAMP was 92.3%, with a specificity of 97.3% and kappa statistics value of 0.84, which is in a strong agreement. *Conclusion.* In this study, LAMP assays coupled with fluorescence dye detection have been found to be suitable for diagnosis and monitoring of *Wuchereria bancrofti* infections in the Kenyan population.

1. Introduction

1.1. Background Information and Literature Review. Lymphatic filariasis (LF) is a chronic parasitic disease of public health and socioeconomic significance in tropical and subtropical countries. It is caused by the filarial worms of *Wuchereria bancrofti*, *Brugia malayi*, or *B. timori* species. This debilitating mosquito-borne nematode infection had been earmarked for elimination by the year 2020 [1]. The disease is transmitted by five genera of mosquitoes, *Culex*, *Aedes*, *Anopheles*, *Mansonia*, and *Ochlerotatus* [2], making it the most common vector-transmitted parasitic infection after malaria [3, 4]. It is the second leading cause of permanent and long-term disability worldwide after eye blindness [5]. Acute and chronic morbidity resulting from lymphatic filariasis has affected 120 Million people living in 81 endemic countries with 1.34 billion people at risk of developing the infections [6]. In 2000, the Global Program to Eliminate LF (GPELF) was launched [7] and it was estimated that 5–6 rounds of mass drug administration (MDA) with ivermectin or diethylcarbamazine and albendazole were required to eliminate the disease [5]. In 2018, 893 million people in 49 countries were living in areas that required preventive chemotherapy to stop the spread of the infections [8].

Early and accurate diagnosis of *Wuchereria bancrofti* causing *bancroftian filariasis* is a key factor in the effort to eliminate filariasis [9]. The diagnosis has relied on the detection of microfilariae in blood specimen and in mosquitoes

Appendix VIII: Abstract Presentation at the 7th East African Health & Scientific conference in March, 2018 at Mwalimu Nyerere Convetional Conference Centre in Tanzania

Title; Molecular Characterization and Phylogenetic analysis of *Wuchereria bancrofti* in human blood samples from Malindi and Tana River Delta, endemic regions in Kenya

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Background; Lymphatic filariasis is a debilitating disease caused by filarial worm *Wuchereria bancrofti*. It is earmarked for elimination by the year 2020 through the Global Program for the Elimination of LF. In Kenya mass treatment has been ongoing since the year 2002 though it has not been consisted as recommended by World health organization. Understanding the genetic differences in *Wuchereria bancrofti* could provide insight into effectiveness of drug regimes, the optimal time-course of drug administration and the potential of development of drug resistance. Data on genetic characterization of *W. bancrofti* in Kenya is lacking. This study, therefore aimed at determining *W. bancrofti* genetic diversity in Kenya.

Methodology; *Wuchereria bancrofti* genomic DNA was extracted from 100 human blood samples obtained from Mpirani district in Malindi and Kipini district in Tana River Delta, amplified by PCR and detected through gel electrophoresis. Seventeen PCR products were purified and quantified for Sanger sequencing. Chromas version 2.6.5 and BioEdit softwares were used for sequence alignment and editing. Fourteen sequences were selected for analysis by MEGA 7. Intrapopulation, interpopulation diversity and pair wise distance were calculated and the phylogenetic trees constructed. Tajima's D test of neutrality was also determined.

Results; the mean diversity of Malindi and Tana River Delta isolates was 0.75 and the overall mean distance was 0.99. Tajima's (D) test for test of Neutrality was 4.149 and nucleotide diversity(π) 0.603.The phylogenetic tree reconstruction showed that the

sequences clustered in to 3 different clusters. The evolutionary rate between sequences was P= 0.00226, df=1, $p<0.05$. On NCBI blast, the sequences had an identity of 79-98%

Discussions; There was a great divergence observed within Malindi population (0.95) compared to that of Tana River Delta (0.4). Genetic distance was evaluated, Tana River had smaller distance (mean 0.4) compared to Malindi (mean 1.81) and thus Malindi had higher divergence compared to Tana River isolates. The phylogenetic tree reconstruction showed that the sequences clustered in to 3 different clusters. This indicated that there were 3 different strains in the study population. The Neutrality and selection test results showed that strong selection was occurring in Wuchereria bancrofti populations.

These results reviewed high genetic variations of *W. bancrofti* in Kenya. This variation may be attributed to prolonged use of the mass drug administration and the long period of parasite circulation in the population. P value less than 0.05 and this made us conclude that there were differences in lineages of all the sequences.

Conclusions and Recommendations; this study provides information on population diversity and structure of *W. bancrofti* in Kenya. Knowledge of the population structure and genetic differentiation of this parasite provides important insights into patterns of transmission, disease outcome, drug resistance, and influence the design and implementation of public health interventions. We recommend that more studies on genetic diversity be carried out to all endemic regions in Kenya. The data adds to our understanding of the phylogenic diversity of these devastating parasites and the genetic information could support the control and monitoring of LF in these endemic areas.

Appendix IX: Abstract at the 11th KEMRI Annual Scientific Health Conference (KASH) in June 2021 at Safari Park Hotel in Nairobi, Kenya

Design, Optimize and Compare Loop Mediated Isothermal Amplification and Helicase Dependent Amplification Assays, for point-of-care detection of *Wuchereria bancrofti* DNA in human blood in Tana River Delta, Costal- Kenya

Authors:

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Introduction:

Diagnosis of *W. bancrofti* causing bancroftian filariasis has relied on the detection of microfilariae in blood specimen and mosquitoes. Point-of-care diagnosis of lymphatic filariasis is largely based on microscopic examination and morphological assessment of stained microfilariae from blood collected at night (2200pm-2000am) to coincide with the nocturnal periodicity of the microfilaria (WHO, 2011). Circulating filarial Antigen testing by Immunochromatographic card test (ICT) was described by WHO as the Gold standard but has been found to have lost its sensitivity and also cross-reactivity with other filarial parasites have been noted. Molecular assays though used, they are not routinely available for the diagnosis of filariasis in most clinical or reference laboratories but are available at specialized research centers and public health laboratories (Blaire *et al.,* 2019). Isothermal assays that relay on a constant temperature such as Loop-mediated isothermal amplification (LAMP) and Helicase Dependent amplification assays are also used in detection of pathogens causing diseases. A number of studies by Poole *et al* (2019), Drame *et al.,* (2014) and Takagi *et al*, (2011) among others have described use of these assays, however the assays have not been validated for use in most of the endemic regions. Due to

many limitations of the current diagnostic tools for *W. bancrofti*, such as lack of sensitivity of ICT and the cross- reactivity and the need for sophisticated equipments and trained personnel by PCR, novel diagnostic methods that are simple, rapid, sensitive, and reliable are required.

The General objective of this study was to evaluate LAMP and HDA as molecular Isothermal Amplification assays for diagnosis of *Wuchereria bancrofti* to aid in monitoring the prevalence of the disease in Kenyan endemic areas. Here, we tested the specificity, sensitivity and the Kappa statistics of the Isothermal Methods against conventional PCR as a 'Gold standard'.

Materials and methods

Approvals for sample collection were sought from KEMRI-SERU and the participating County authorities. Samples for this study were collected from Tana River Delta in Tana River County, in Kenya. Participants were recruited on voluntary basis and 4ML of blood drawn after consenting. The samples were taken to KEMRI Filariasis unit for processing. 200ul of each Samples were subjected to DNA extraction by Alcohol precipitation method as described by Datta *et al*., (2007). Specific primers for each method were used targeting 18srRNA species specific regions (Ssp1) on *W. bancrofti* complete sequence accession no. AY297458 yielding 188base pairs. Amplifications were done by LAMP, HDA and PCR. Detection was done on intercalating dyes or Gel electrophoresis. Sensitivity, specificity and Kappa statistics were estimated by 2X2 contingency table and compared.

Results;

Each of the125 samples obtained were amplified by the 3 methods. The study showed positive cases of 13, 15,18 representing infection rate of 10.4%, 12% and 14.4% by PCR, LAMP and HDA respectively. The sensitivity of LAMP was 92.3% and that of HDA was 76.6%. Specificity for LAMP was 97.3% while that of HDA was 93.7%. Kappa statistics was at 0.84 and 0.67 for LAMP and HDA respectively.

Conclusion: LAMP assay was found to be more comparable to PCR and thus, it can replace PCR in field settings for diagnosis of *W. bancrofti* in Kenya.

Recommendations; We recommend more validation studies to be carried out in other endemic regions including a non-endemic region for conclusive recommendation for use of LAMP methods.

Appendix X: HDA Primer3 Output on *Wuchereria bancrofti* Sequence

Primer set 1
>Forward
TATTGATAGTGTACGGGAGGGGTTTGG
>Reverse
GCTTCAGGCTTCTCTCATTTCACCACT

PRIMER PICKING RESULTS FOR Wuchereria bancrofti 18s
OLIGO start len tm gc% any 3' seq
LEFT PRIMER 21 27 67.51 48.15 4.00 0.00 TATTGATAGTGTACGGGAGGGGTTTGG
RIGHT PRIMER 117 27 67.96 48.15 3.00 1.00 GCTTCAGGCTTCTCTCATTTCACCACT
SEQUENCE SIZE: 1579
INCLUDED REGION SIZE: 1579

PRODUCT SIZE: 190, PAIR ANY COMPL: 4.00, PAIR 3' COMPL: 1.00
PRODUCT Tm: 73.1606, PRODUCT Tm - min(OLIGO Tm): 5.6528
1 GTAAGAGGAAAAAGATAGTGTATTGATAGTGTACGGGAGGGGTTTGGGCGTATGTTAGGT
>>>>>>>>>>>>>>>>>>>>>>>>>>
61
ATAGGTGATGTGAGGATGATAGTTCGGAGGAGTGGTGAAATGAGAGAAGCCTGAAGCGCA
<<<<<<<<<<<<<<<<<<<<<<<<<<
121
AGCATAGCAAAGTCAACTCCTGCACGCGGACGGGACATAACCAGTGATAAAGGCGGATAT
181
AAAGCCCAACCGGGACCTGGACCTCCACCACTAAACAGTGAGAGAACGAGCAAAAGAAGA
241
GACGACGGTAGGAGCCAGAAGCGCAGGGGAGTCAGGCGGGGAGAAGCCGGCTCGGTGCGC
301
CAATCCTGAGAGGGACAGAACCAGTGAAGAATCCTCCCCTGAGGGCCGGGTCCCCCTCCC
361
CGGCCCCAAAGGAGCGGAAGGGAAATGTTTTTTAATATTTTCAAGGATGAAAGGAATTTT
421 TAGCAATTTTTTTGGTTATATTTTTATTTGAATTATTTTTTTTTTTTGTTTGCTTGGTAT
481 AACCTTATTTTTAATCTTTTTTATGTCAGTCGCAAGCATCGGATAGGTATCAATCAGCGC
541
AACGGTGTGAGCGAGACAGACCAAACAAACAGTGGCATACAGTTAACGACAGTCCTTTTG
601
CGCGCCTGTTAGGTATAGTAACTATAAAGTGGATCGCCCCAGGAATTGATGACATACCAG
661
CAACGTGAAGTGCAAGTATAGCAATGTCAACTCCTGCACTCGGATGGGACATTACCTGTG
721
ATAAAGGAGGATATAAAGTCCAACCTGTACCTGGACCTTCACCAATAAACAATGAGAGAA
781
TGAGCAAAATAAAAGATGACACTAATAACCAAAAACTTAAATTATTCATACGAGGAAATG
841
CCATATCTGGTGCGCCAATCATGAGAGGTACAAACCAGTTACCAAATCCTCCCATGAGGG
901 CTGGCATTATCTCCTGGCATCAAAGTAGCGTAAGTGAATTGTTTTTTAATATTTTCAAG
961 ATGAATGGAATTTTTAGCAATTTTTTTGTTTATATTTTTATTTGAATTATTTTTTTTTTT
1021 TGTTTGCTTGGTATAACCTTATTTTTAATCTTTTTAATTTTAGTAAAAAGAGTATATAGA

1081
GAATGTAATAGTAGTAAAAGGAAAATGTTGAGAGTGGATGTTAAATATTATAGATATAGT
1141
GTTAATATGATATGGTATTTAGTAAAAAAAAGAAGAGTGAAGGGTGAGAAAAGAAAATAA
1201
AAATAGTAGTAAAGGAAGGAAGTAAAAAAGTGGAAAATGGAGGATATAAAAGAGGAAAAT
1261
GTATATGGATATATGTCAGGAAAAGTATGAGAGAAAGAGTAAAAGAAAAGATGATGATAA
1321
AAAGAAAAAAAAGGTGGGGATGAATGGGGGGAAAAGGGGGAGAGGGAGAGAAAATGTTGAG
1381
AGGAAAAAATGAGAAAAAAAGGGTGTGTTGAGGGAAGGGATTTTTCTTCGTGGGATTAAA
1141 ATAGGGTAAGGGAATTGTTTTTTAATATTTTTAAGTATGAATGGAATTTTTAGCAATTTT
1501 TTTGTTTATATTTTTATTTGAATTATTTTTTTTTTTTGTTTGCTTGGGTATAACCTTATT
1561 TTTAATCGTGTTATTGATC