

**THE INFERENCE OF IMMUNOCHROMATOGRAPHIC TEST, MICROSCOPY
AND POLYMERASE CHAIN REACTION IN DIAGNOSIS OF LYMPHATIC
FILARIASIS IN TANA DELTA, KENYA**

By

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**A research thesis submitted in fulfillment of the requirements for the award of
degree of Doctor of Philosophy in the School of Pure and Applied Sciences,
Kenyatta University.**

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DECLARATION

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

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DEDICATION

This thesis is dedicated to my beloved family, Teresa, Ruth, Oren and especially my late mother, retired Elder Mary Wangari Kagai.

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ACRONYMS AND ABBREVIATIONS

AD	- Ano domino
ADL	- Acute Adenolymphangitis
ADLA	-Acute dermatolymphangiadenitis
AFL	- Acute filarial lymphangitis
ANOVA	-Analysis of Variance
ATP	-Adenosine triphosphate
BSA	- Bovine Serum Albumin
CDC	- Centre for Disease Control
CFA	-Circulating filarial antigen
DALYs	- Disability adjusted life years
DEC	- Diethylcarbamazine
DNA	- Deoxyribonucleic Acid
dNTPs	- Deoxynucleotides
EDTA	-Ethylene diamine tetraacetic acid
ELISA	- Enzyme-linked immunosorbent assay
GPELF	-Global Program for the Elimination of Lymphatic Filariasis
HCl	- Hydrochloric acid
ICT	- Immunochromatographic test
IL	- Interleukin
Ig	- Immunoglobulin
KEMRI	- Kenya Medical Research Institute
LDR	-Long DNA repeat

L1, L2, L3	- larval stage of <i>Wuchereria bancrofti</i>
LF	-Lymphatic filariasis
MDA	- Mass drug administration
NaOH	- sodium hydroxide
NH ₄ OAc	- Ammonium Acetate
NPELF	-National program for elimination of lymphatic filariasis
MgCl ₂	- Magnesium chloride
PCR	-Polymerase chain reaction
PVP	- Poplyvinyl pyrrolidine
RAPD	- Random Amplified Polymorphic DNA
RAPLOA	- rapid assessment procedures for loiasis
RFLP	- Restriction Fragment Length Polymorphism
RNA	-Ribonucleic Acid
RPM	- Revolutions per minute
SDS	- Sodium Dodecyl Sulphate
SSC	- Sodium Citrate-Sodium Chloride
SSP	-Species specific primer
TAE	- Tris acetate
TE Buffer	-Tris-EDTA buffer
TPE	- Tropical Pulmonary Eosinophilia
UV	-Ultraviolet
WHO	-World Health Organization
µm	- Micro-metre

ABSTRACT

The current diagnostic tests for *Wuchereria bancrofti* infections are insensitive and logistically unsuitable in epidemiological studies and disease surveillance. Although needing refinement, potentially sensitive polymerase chain reaction (PCR) assays have been developed. The suitability of these assays as molecular epidemiological and surveillance tools has not been extensively studied. These assays can be used to estimate disease indices, such as vector infection rate and are important in estimation of the infection pattern in the suspect areas. The main objective of this work was to study *W. bancrofti* infections in an identified population in the Tana Delta district, using modified molecular assays. The Tana delta, situated along the Kenyan north coast lies in a potentially lymphatic filariasis endemic region but no data on the disease has been documented. This study improved and evaluated PCR assays in monitoring of *W. bancrofti* infections in humans and the vectors in the Tana Delta. Samples which included, blood, sputum and urine were collected from consenting participants along with mosquitoes which were collected from their homes. The mosquitoes were identified and examined for *W. bancrofti* infections. Observations from the study indicated that PCR assays had sensitivity and specificity above 84%. This study demonstrated that the *W. bancrofti* infections exist in the Northern part of the Kenyan coast with prevalence of 6.4% to 9.6% using traditional diagnostic tests and PCR assays respectively. The prevalence of *W. bancrofti* in mosquito vectors was 1.0% microfilariae by dissection and 1.6% by PCR mosquito assay. A relationship between LF prevalence in the population and vector prevalence was observed. Through a calculation of a factor ψ (Hebrewshin), the relationship could enable projection of LF prevalence in the population from the determined vector prevalence. The study suggests that this factor ψ would be directly proportional to the disease prevalence in the population ($p=0.005$). By using the modified PCR assays which are both sensitive and specific ($p<0.005$), an end point (denoted as a prevalence of 0.1%) for the mass drugs administration (MDA) can be determined. The age group 11-20 years was found to be more enthused in participating in the study, with the best turn out of 31% compared to all other age groups ($p=0.001$). This group had the highest lymphatic filariasis prevalence too. The results from sputum and urine are comparable, suggesting that either of these samples may be used in LF surveillance studies. It is observed that whereas the molecular methods are sensitive and specific, the cost involved is high. The study therefore recommends the use of PCR assays in determination of end-point for MDA. Further, it is recommended that where a survey of lymphatic filariasis is required; 11-20 age group be used to represent the community.

CHAPTER ONE: INTRODUCTION

1.1 Background

Lymphatic filariasis is one of the most debilitating scourges among all parasitic diseases. This disease is caused by three nematoid parasites of *Wuchereria* and *Brugia* genus. The life cycle of these parasites involve mosquito and human hosts. The disease has a wide spectrum of clinical manifestations, most of which are asymptomatic (Beaver, 1970). In the symptomatic forms there is enlargement of the patient's lower and/or upper limbs or genitalia (Barry and Marquardt, 1996). Whereas the disease may not kill the victim, there is severe disfiguration and disablement, which cause enormous losses of social and economic production of the affected persons and communities (WHO, 1998).

Global Program on Elimination of Lymphatic Filariasis (GPELF) was set up by WHO in 1998, and most of the affected countries, Kenya included (Njenga and Wamae, 2001), have formed their national programs for elimination of lymphatic filariasis under the ministries of health. Among the research needs of GPELF is development of surveillance tools, for epidemiological assessment and mapping as means for monitoring the effectiveness of disease elimination interventions (WHO, 1998). One way of carrying out surveillance of lymphatic filariasis, is mass diagnosis of the population. Current diagnosis tests are insensitive or logistically unattractive in cases of such mass diagnosis (Wamae, 1994). A need therefore exists for research into more appropriate tests that are both sensitive and specific for diagnosis of lymphatic filariasis.

An alternative surveillance strategy is assessment of *Wuchereria bancrofti* infections in the vector, mosquito. Determination of infection in mosquitoes has hitherto being carried out by dissection (Ramzy *et al.*, 1995). The accuracy and precision with which *W.*

bancrofti infections in vectors can be estimated is however, limited by the ability to process sufficient numbers of mosquitoes by dissection, particularly when vector infection rates are low (Fischer *et al.*, 2002). PCR assays for mosquitoes could solve this problem posed by dissection.

In this study, PCR assays utilizing body fluid samples of sputum, urine and serum were modified and used for detecting bancroftian filariasis. Prevalence of lymphatic filariasis in both vectors and humans were established and compared in the Tana delta region of the north coast, Kenya.

1.2 Problem statement

There is currently a challenge in diagnosis of lymphatic filariasis. The traditional methods require microscopy of night collected blood, between 2200 and 0200 hours (Dennis *et al.*, 1976, Denham *et al.*, 1977, Knots, 1939). This is logistically challenging in cases of mass diagnosis, and in addition, non-compliance by the communities involved can be high, as people are sensitive to being bled (Lucena *et al.*, 1998). This includes people in the Tana Delta. The antigen test recommended by WHO uses day blood (Weil *et al.*, 1997) but it has shortcomings such as insensitivity in cases of low microfilarimae (Pani *et al.*, 2000, Schuetz *et al.*, 2000) and may not be useful for surveillance in the post elimination phase (Schuetz *et al.*, 2000).

1.3 Justification

Geographical determination of the spread of lymphatic filariasis in an area requires elaborate and effective mass diagnosis of *W. bancrofti* infection. Further, accurate establishment of treatment dynamics, re-infections and new infections are essential in assessment of the disease management strategies (Figuroa, 1998). The need to carry out research aimed at finding better methods for surveillance of *W. bancrofti* infections has been identified (WHO, 1998) in both the vectors and human populations (Ramzy, 2003). This study sought to compare the traditionally diagnostic methods with molecular diagnostic assays to establish prevalence of lymphatic filariasis in both mosquitoes and the population in the Tana delta.

1.4 Research questions

1. Can sputum, urine, serum and mosquitoes be used in PCR assays for mass diagnosis of *Wuchereriabancrofti* infections in both humans and mosquitoes?
2. Can the sputum, urine, serum and mosquito PCR assays be used for lymphatic filariasis surveillance during the elimination process?

1.5 Hypotheses

Sputum, urine and mosquito samples can be used in PCR assays for mass diagnosis of *Wuchereriabancrofti* infections.

Data from the above PCR assays can be used for *Wuchereriabancrofti* infection surveillance.

1.6 Objectives

1.6.1 Main objective

The main objective was to perform a molecular epidemiology of *Wuchereriabancrofti* infections in the Tana Delta, Kenya involving; *W. bancrofti* infections in mosquitoes and a defined population using modified molecular assays.

1.6.2 Specific objectives

- i). To determine *W. bancrofti* infection prevalence in a population in Tana Delta District using the modified PCR assays for sputum, urine, and serum.
- ii). To determine *W. bancrofti* infection prevalence in a population in Tana Delta District using microscopy.
- iii). To determine *W. bancrofti* infection prevalence in a population in Tana Delta District using immunochromatographic test (ICT).
- vi). To determine *W. bancrofti* infection prevalence in mosquitoes found in the Tana Delta District using microscopy and modified PCR assay for mosquitoes.

CHAPTER TWO: LITERATURE REVIEW

2.1 Filariasis

Filariasis is an infectious tropical disease that is caused by thread-like roundworms belonging to the superfamily Filarioidea. There are eight known filarial nematodes which use humans as their definitive host. These are divided into three groups according to the part within the body that they occupy (Taylor, *et al*, 2010). These worms are known to occupy: the lymphatic system, subcutaneous tissue and serous cavity. Lymphatic filariasis is caused by three worms; *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori*. These worms occupy the lymphatic system, including the lymph nodes. Subcutaneous filariasis is caused by *Loa loa* also known as the African eye worm.

The worms that occupy the subcutaneous fat layer of the skin include *Mansonella streptocerca* and *Onchocerca volvulus* (Noma, *et al*, 2002). Serous cavity filariasis is caused by the worms *Monstella perstans* and *Mansonella ozzardi*, which occupy the serous cavity of the abdomen. In all cases, the transmitting vectors are blood-feeding arthropods, mainly black flies and mosquitoes. *Onchocerca volvulus* and *Mansonella perstans* occurs in Africa and South America. *O. volvulus* has also been found in the Middle East. Among the other species, *Loa loa* and *Mansonella streptocerca* are found in Africa. *Mansonella ozzardi* occurs only in the American continent. Among the agents of lymphatic filariasis, *Wuchereria bancrofti* is encountered in tropical areas worldwide; *Brugia malayi* is limited to Asia; and *B. timori* is restricted to some islands of Indonesia.

2.2 Lymphatic filariasis

Commonly known as elephantiasis, lymphatic filariasis has a long history dating back beyond 5000 years (Holdrich and Jones, 1983; Smith, 2004). The disease is a syndrome caused by an obstruction of the lymphatic vessels, which results in extreme swelling of the skin and tissues primarily affecting the limbs and genitals (King and Freedman, 2000; Wright and Baird *et al*, 1974). Although non-parasitic forms of elephantiasis such as podoconiosis (Destas *et al*, 2003) exist, *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*, filarial worms belonging to the phylum Nematode are the causative agents for the parasitic lymphatic filariasis. These parasites are transmitted by over seventy seven species of mosquitoes which have been identified to date. *Wuchereria bancrofti* causes more than 90% of lymphatic filariasis cases worldwide (Ottesen *et al*, 1990). The disease is a major cause of disfigurement and disability in the endemic areas, leading to significant economic and psychosocial impact. Estimates suggest that, of the 120 million infected people more than 40 million are seriously incapacitated and disfigured by the disease (Figueroa, 1998). The diagnosis of lymphatic filariasis has been difficult (Wamae, 1994) as the available methods such as clinical observations, microscopy and serology tend to have poor sensitivity and/or specificity. The endoparasite *Wuchereria bancrofti* which appears to infect only humans (King and Freedman, 2000) releases live young larva called 'microfilaria' into the blood stream (Figure 2.1) after maturing in about nine months. These microfilariae get ingested by mosquitoes when they feed on an infected person. Inside the mosquito they live and develop until the third larval stage known as L3 and wait for the mosquito to bite another host whereupon they enter the host via the mosquito's proboscis sheath and the wound it makes in the host's skin (Manson-

Bahr and Apted, 1984). Adult worms live for over five years, and during their life in the host may block the host's lymphatic system, hence developing elephantiasis.

2.3 Zoonotic filariasis

Filariasis of animals, especially those of mammals, often infect humans and typically produce cryptic infections (Orihel and Eberhard, 1998). These "zoonotic" infections have been reported from virtually all parts of the world including temperate zones. Infections may be symptomatic or not, and the parasites are found in surgical tissue biopsy specimens or, more rarely, are removed intact from superficial sites such as the orbit or conjunctiva. Typically, these worms tend to occupy tissue sites similar to those occupied in the natural animal host, with the exception of the eyes (Gutierrez, 1990). Many kinds of filariae have been isolated from humans, including species of *Dirofilaria*, *Brugia*, *Onchocerca*, *Dipetalonema*, *Loainia* and *Meningonema*. Worms have been found in subcutaneous tissues, the heart and lungs, lymphatics, the eye, and the central nervous system.

All of the filariae utilize bloodsucking insects, such as mosquitoes as biological vectors, so that humans are infected by zoonanthrophilic species which fed previously, in an appropriate time frame, on an animal with a patent filaria infection (Anderson, 1992). Specific identification of these filariae is based on their morphological features in histologic sections. Unfortunately, some of these worms cannot be identified even at the generic level. There are other species of filariae, presumed to be zoonotic, which produce patent infections in humans but are poorly and incompletely known (Orihel and Eberhard, 1998). These include *Microfilaria semiclarum* and *Microfilaria bolivarensis*. It is

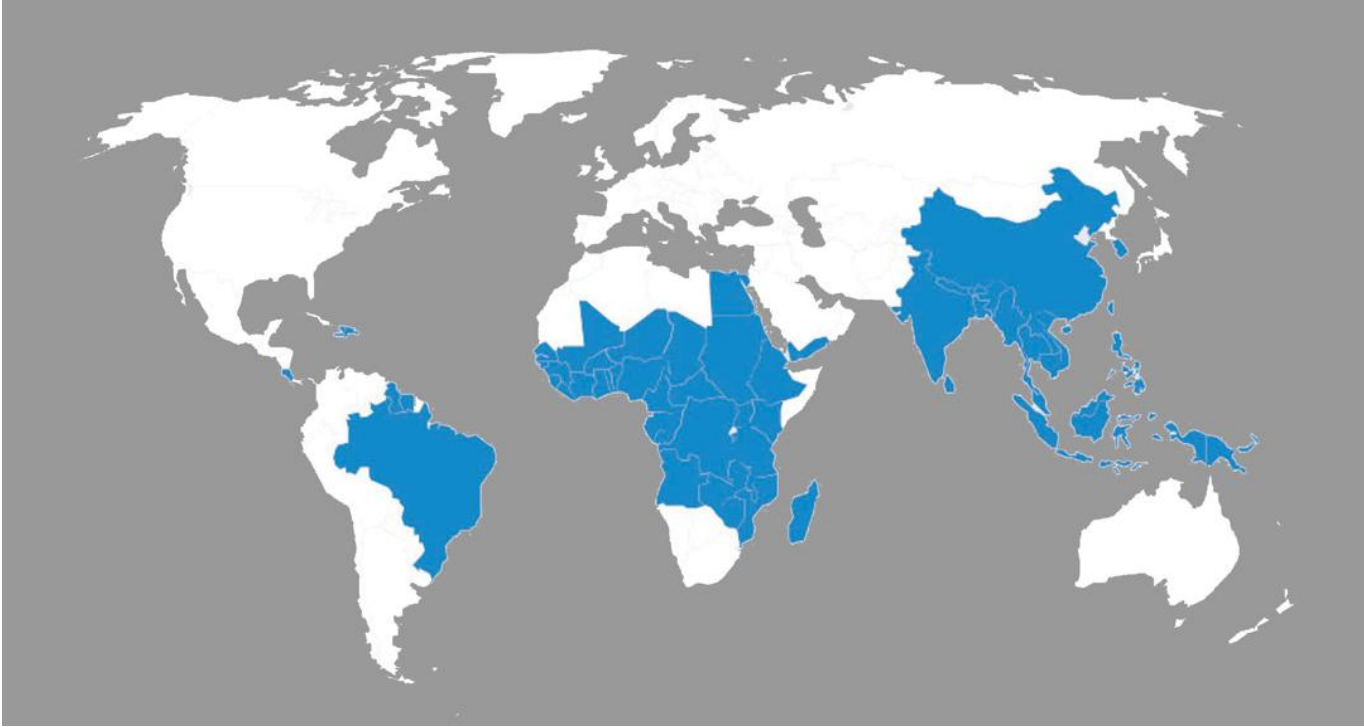
probable that almost any filaria parasitizing animals can, under proper circumstances, infect humans and undergo some degree of development. When these parasites die in the tissues, the host mounts a foreign body response to their presence. It is unclear in these cases whether the parasite becomes moribund and the host responds to the dying worm or whether the host ultimately mounts a response which kills the worm. Inasmuch as most of these infections persist for months without a detectable host response, it seems likely that at some level, the worm finds itself in an unnatural host and succumbs and that this is followed by a tissue reaction to the dying worm. This argument is further strengthened by the observation that in their natural hosts, filariae are typically long-lived, living often several years or more. Zoonotic infections are typically cryptic; meaning, only in rare instances are circulating microfilariae found (Nozais, et al, 1994).

The zoonotic filariae recovered most commonly from humans are found in the subcutaneous tissues; the majority belongs to the genus *Dirofilaria*. The dirofilarias are natural parasites of a variety of animals; they live in the subcutaneous tissues of their hosts, produce microfilariae which circulate in the blood and in most instances are transmitted by mosquito species. Development in the mosquito requires about two weeks; these parasites require several months to reach sexual maturity in their natural definitive hosts (Orihel, 1969).

2.4 Geographical distribution of lymphatic filariasis

Lymphatic filariasis is found along the tropical zone and is endemic in large parts of Asia, Africa and South America (Figure 2.1). Together with some Pacific islands and other sub-tropical countries there are a total of 83 countries which are endemic with lymphatic filariasis. The disease affects 120 million people worldwide (Palumbo, 2008). It

is estimated that 1.2 billion people are at risk of infection worldwide (Wijers, 1977a; Michael and Bundy, 1997). Over 33% of the infected suffer from the overt symptoms such as elephantiasis and hydrocele. Of the three parasites that cause lymphatic filariasis, *Brugia malayi* occurs mainly in China, India, Malaysia, the Philippines, Indonesia, and various Pacific islands whereas *Brugia timori* is limited to the Timor Island of Indonesia (Manson-Bahr and Apted, 1984). *Wuchereria bancrofti* infections occur in Africa, Southeast Asia, the Indian subcontinent, the Pacific islands and Latin America (Figure 2.1). Within endemic regions, the infection has a focal distribution that coincides with areas conducive to breeding sites for the mosquito vector. Thirty nine African countries are endemic for lymphatic filariasis (Michael and Bundy, 1997; Lindsay and Thomas, 2000). These countries include Zimbabwe in the south to Niger and Egypt in the north. The disease is also found Burkina Faso, through Democratic Republic of Congo in the west and Kenya in the east. Lymphatic filariasis is endemic in the Islands of Zanzibar and Seychelles in the Indian Ocean and Sao Tomé and Príncipe in the Atlantic Ocean (Menendez and Bouza, 1988, WHO, 1998). In Kenya, bancroftian filariasis is endemic in the coastal districts with an estimated population of 2.5 million people being at risk of infection (Wijers, 1977a; WHO, 1998; Njenga and Wamae, 2001).

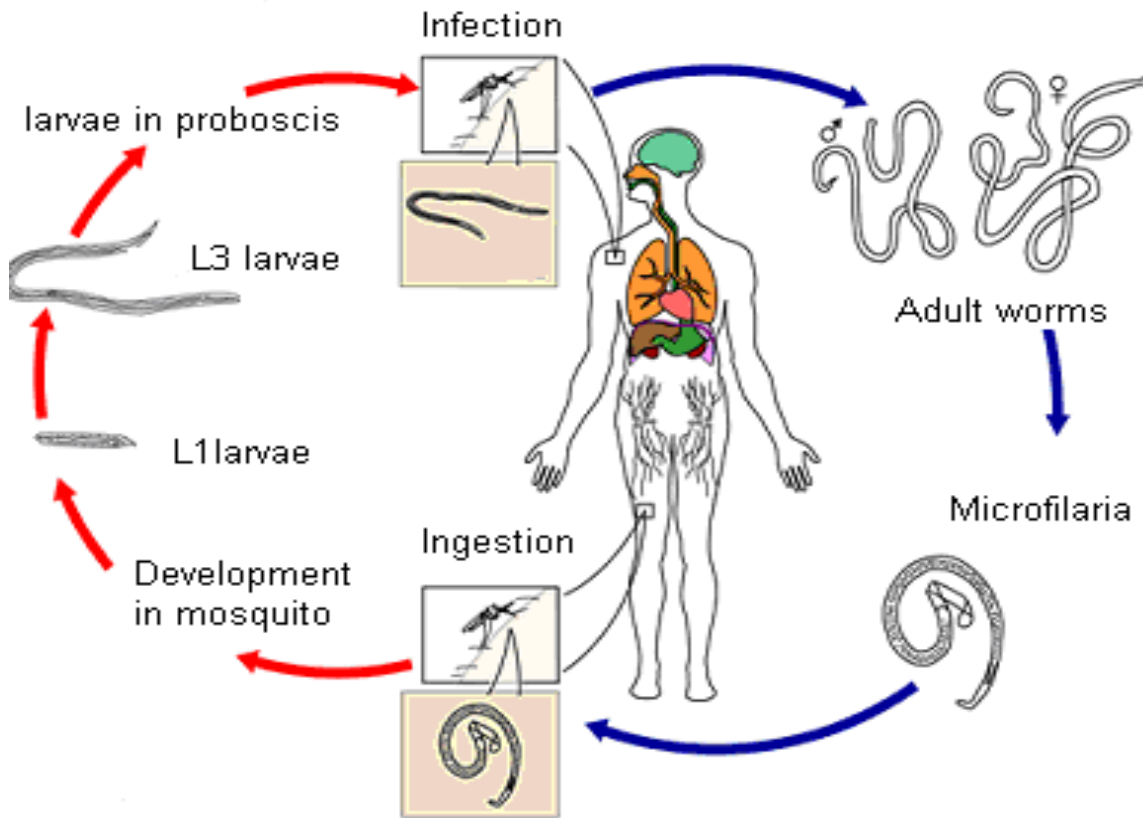


Key: Blue= lymphatic filariasis endemic countries

Figure 2.1: Geographical distribution of lymphatic filariasis (source; WHO)

2.5 Life cycle of *Wuchereria bancrofti*

The life cycle of *Wuchereria bancrofti* oscillates between man and the vector mosquito, the man being the definitive host (Figure 2.2). Once an infected mosquito deposits viable L3 larva, they quickly travel through blood capillaries and veins to the lungs, where after some unspecified time move back to circulation and penetrate through tissue into the lymphatic vessels. The larvae mature sexually and physically increase in size to a maximum of 60mm incases of males and up to 100mm incases of females (Table 2.1). Adult females are viviparous and produce thousands of microfilariae, which can be seen in the patient's blood between 22.00 and 02.00 hours due to their nocturnal periodicity.

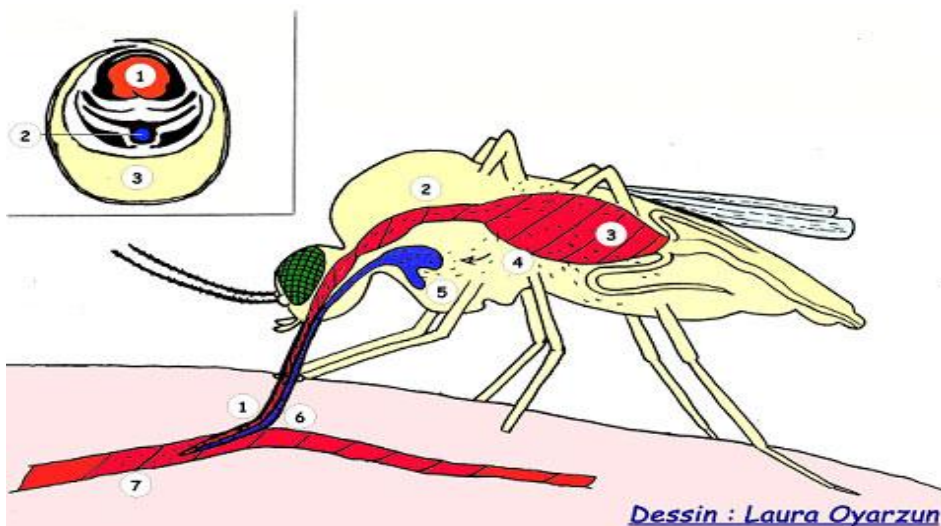


Source:WHO

Figure 2.2: The life cycle of *Wuchereria bancrofti*

The female mosquitoes require mammalian blood for production of eggs. The mosquitoes are attracted to mammals, including humans through body odors, released carbon dioxide and body heat, during breathing and/or sweating (Carey *et al*, 2010). The mosquitoes become infected after a blood meal from an infected person (Figure 2.3). The microfilariae in the mosquito gut lose their sheaths, and travel from the gut to thorax, head and proboscis in 10 to 40 days depending on the environmental temperatures (Manson-Bahr and Apted, 1984). After losing the sheath in the gut they become plump or sausage shaped, and they are referred to as L1 larvae (Beaver *et al*, 1984). The larva becomes elongated, slender and changes to L2 and then L3 forms. The L3 larva is the

infective stage and is found in the proboscis, (Simonsen and Dunyo, 1999). Upon transmission to man through a mosquito bite, the L3 larvae migrate from sub-cutaneous tissue, to blood vessels and penetrate the lymph vessels where they mature and localize (Beaver *et al*, 1984). The adult worms live intertwined together and after mating, produce microfilariae which in-turn are taken up by mosquitoes to perpetuate the life cycle (Manson-Bahr and Apted, 1984). Large uncontrolled populations of vectors may sustain transmission or contribute to rapid resurgence (Chadee *et al*, 2002) of lymphatic filariasis after an MDA programs. An efficient method for assessing prevalence of lymphatic filariasis in a population of vectors could permit the xeno-monitoring of elimination program (Ramzy, 2003).



Source: Sandra Laney

Figure 2.3: The life cycle of *W. bancrofti* in mosquito

Infection rate in vectors is an important parameter in determining transmission and it is conventionally assessed by dissection and microscopy. A PCR assay based on

Ssp I repeats of *W.bancrofti* has shown potential in the detection of infection in vectors (Hoti, *et al*, 2001). In her study, Hoti evaluated the specificity and sensitivity of the assay on *W. bancrofti* and its vector, *Culex quinquefasciatus*. The DNA from pools of *C. quinquefasciatus* to which *W. bancrofti* microfilariae (mf) were added was extracted by lysing with NaOH and sodium dodecyl sulphate (SDS), followed by silica absorption in the presence of guanidinium thiocyanate. The assay was carried out using NV-1 and NV-2 primers and the species specific SspI band was visualized on agarose gels stained with ethidium bromide. The assay was found to be highly species specific, as it did not detect the DNA of a closely related filarial parasite, *Brugia malayi*. Minimum number of parasite detectable in pools of mosquitoes was 1 mf. The assay was found to be highly specific and sensitive in detecting filarial parasite in pools of mosquitoes.

The main mosquito genera involved in transmission of lymphatic filariasis are *Anopheles*, *Culex* and *Aedes* (Manson-Bahr and Apted, 1984). Seventy seven species belonging to the four genera have been identified as LF transmitters (Manson-Bahr and Apted, 1984). Culicine and anopheline mosquitoes are the main vectors of the nocturnally periodic forms of *W. bancrofti*, found in most of Africa, the Caribbean, India and some of the countries and islands surrounding India (Simonsen and Dungo, 1999). The day biting *Aedes polynesiensis* transmit the sub-periodic form of lymphatic filariasis in various pacific islands. Due to the vector preferred feeding habits, lymphatic filariasis demonstrates focal and periodic distribution patterns dependent on behavior of the vector (Manson-Bahr and Apted, 1984). *Mansonia* species have been incriminated in transmission of LF in Asia (Chang, *et al.*, 1993).The possible role of *Mansoniauniformis* mosquitoes in the transmission of lymphatic filariasis was assessed in an endemic area of

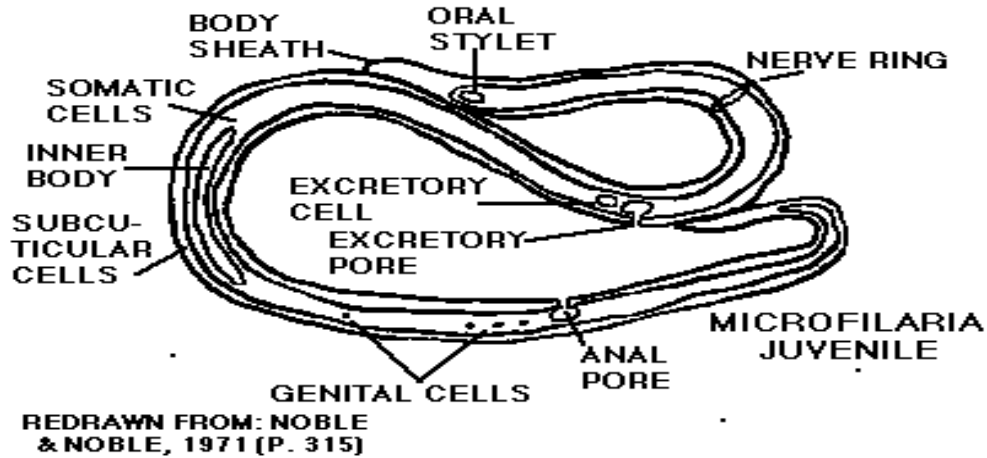
Uganda, by Onapa, *et al.*, (2007), by examining *Mansonia* biting cycle, host preference and ability to support the development of experimental and natural *Wuchereriabancrofti* infections. Experiments revealed that outdoor biting peaks early in the evening (19:00-20:00h), while indoor biting peaks around midnight (23:00-24:00h). Both biting and feeding behavior are compatible with a potential for transmission. Experimental feeding of *M. uniformis*, with microfilariae, demonstrated that the microfilariae can develop from L1 to L3 though in very low quantities (0.7%). Dissection of wild caught *M. uniformis* did not reveal any natural infections with *W. bancrofti* infective larvae, which demonstrates that *M. uniformis* has a limited potential in supporting development of *W. bancrofti* to the infective stage and it does not appear to play a role as a vector under natural conditions.

After several injections of microfilaria by the mosquitoes, adult worms are gradually acquired over the years, slowly accumulating and producing microfilaria in the infected individuals. The prevalence of microfilaria in endemic communities therefore tends to increase with age (Pani and Dhanda, 1994). Lymphatic filariasis is almost entirely a disease of the poor in the community. Lack of basic hygiene due to illiteracy coupled with poor or inadequate housing without basic sanitation expose people to the risk of infection with *W. bancrofti* transmitted by the principal vector, *Culex quinquefasciatus* (Bogh *et al.*, 1998).

2.5.1 The microfilaria

Microfilaria is the larval stage of the filarial worms which can be found in both the definitive and the intermediate hosts. Microfilaria has three stages in its life, designated as L1, L2 and L3. Whereas L1 and L3 can be found in both hosts, L2 is only found in the intermediate host. Immediately after birth, the L1 microfilaria, also known as pre-larvae, find their way into the peripheral blood circulation (Manson Bahr and Apted, 1984). They are unable to live long in the human host and have to be transported to the intermediate host, mosquito.

The mosquitoes become infected after taking a blood meal from an infected person. The microfilariae in the mosquito gut lose their sheaths, and travel through insect muscles, from the gut to thorax, head and proboscis. The larvae take 10 to 40 days to develop, depending on the environmental temperatures. In warm temperatures, 25-35°C, it usually takes approximately 15 days, while it takes longer >30days in environmental temperatures of < 25°C(Manson-Bahr and Apted, 1984). The L1 are taken by the mosquito as it feeds on the host (Beaver *et al.*, 1984). They are transported into the mosquito gut where they change into plump sausage-shaped larvae L2 measuring 1.5mm wide and 2.0mm long. The L2 penetrate the gut muscles of the mosquito and slowly crawl into thoracic flight muscles. After 2 molts, they change into third-stage L3 larvae, which migrate further through the head, eventually reaching the proboscis of the mosquito(Simonsen and Dunyo, 1999). This slender, long shaped larva, L3 is the infective stage of the microfilaria. During the mosquito's next blood meal (Figure 2), infective L3 larvae escape onto human skin and enter through the mosquito bite puncture wound or local abrasions.



Source: Prof. Joseph Hamburger

Figure 2.4: The anatomy of microfilaria

Man is the definitive host of *W. bancrofti*. The third-stage infective larvae, L3, enter the blood through the wound made by the mosquito. They then migrate to the nearest lymph gland where they mature into the thread like adult worms about 3 months to 1 year later. The average incubation time before patency is about 15 months. Adult worms may live in humans for 10 to 18 years, continually producing thousands of microfilariae per day. The damage of the lymphatic vessels caused by the parasites may trigger the immune system's responses noticed in the various lymphatic filariasis patients (Manson-Bahr and Apted, 1984). The microfilariae of *W. bancrofti* can be identified in blood smears by their sheath, size and the anterior nucleus near the head and posterior nucleus near the tail. Once male and female mate the female viviparously produces microfilariae (first stage larvae or L1) which then move through the circulatory system

and collect in arterioles of the lung during the day and emerge at night (nocturnally periodic) when night biting mosquitoes are most active.

2.5.1.1 The microfilarial periodicity

The microfilariae exhibit a phenomenon in which they appear in the periphery blood of the host at certain times during the 24 hour day (Hawking, 1967). In nocturnal periodicity, the parasite appears in blood between 2200 and 0200 hours in the morning. In one study conducted in Brazil by Fontes *et al*,(2000) all patients who had detectable microfilaraemias from 2300 hours to 0600 hours, no microfilariae could be detected in smears prepared from samples collected at 1500 hours from the same patients. In the study, maximum microfilarial densities occurred at 0051 hours. Similarly, when the microfilariae appear between 1000 and 1600 hours during the day, it is called diurnal periodicity. Nocturnal microfilariae of *W. bancrofti* appear in blood circulation at midnight \pm 2 hours, whereas *Brugia malayi* mostly displays diurnal periodicity, in which the microfilaria appear in the blood circulation from 1000 to 1600 hours. In diurnal sub-periodicity, parasites appear in blood circulation at all times but the microfilariae population is higher at noon. Nocturnal sub-periodicity, parasites appear in blood circulation at all times but population is higher at midnight. In ‘aperiodic’ microfilaraemia, the parasite does not appear in blood circulation at all. According to Denham and McGreevy, (1977), filarial parasites tend to have differing behavior while in different regions of the world. In East Africa, *W. bancrofti* nocturnal periodicity may change to be diurnal if the patient sleeps during the day and becomes active at night. *Brugia malayi* displays at least three forms of periodicity. Experiments on the periodicity,

suggest the parasites' preference to high oxygen tension, in which case the parasites are to be found in the lungs where there is high oxygen tension during the day but in the peripheral blood at night. When the difference between venous and arterial blood oxygen tensions is under 53mmHg, microfilariae will leave the lung for the general circulation and hence appear in the peripheral blood. When the difference is greater, they accumulate in the lungs. Often periodicity may be affected by the geographical zones where the patient is living. In Malaysia *Brugia malayi* displays both nocturnal and diurnal types of parasites, whereas the Philippines display nocturnal and sub-periodic types. There is a wide distribution of nocturnal and diurnal types of parasites in the Indian sub continent, especially in the regions to the east of Kerala (Holdrich and Jones, 1983). When exposed to different hosts, *Brugia malayi* nocturnally periodic in humans, changes to sub-periodic when introduced into cats. The sub-periodic form remains the same in both cat and human but is nocturnally periodic in some species of monkeys (Denham and McGreevy, 1977). Periodicity of microfilariae has more to do with the worm responding to human diurnal physiological changes (Denham and McGreevy 1977). The parasite's bio-rhythm follows the host's bio-rhythm but with a time-lag of more than a week. A nocturnally periodic *W. bancrofti* (as found in East Africa for instance) becomes diurnally periodic if the individual sleeps by day and is active by night. These microfilariae possess a photosensitive substance containing a vitamin-A-like carotenoid in fluorescent granules in the epidermis which causes them to leave the peripheral circulation in day light and collect in lungs (Denham and McGreevy, 1977). Periodic microfilariae possess numerous granules, when compared to aperiodic forms which have few or none. Periodicity can vary for the same parasite in different hosts. Thus *B.*

malayi has three forms (at least). In humans, one is nocturnally periodic, another nocturnally sub-periodic and the third is diurnally sub-periodic. That which is nocturnally periodic in humans, becomes sub-periodic when injected into cats (Manson Bahr and Apted, 1984). On the other hand, the sub-periodic form is sub-periodic in both cats and man but is nocturnally periodic in two species of monkeys.

Table 2.5: Various measurements of filaria parasites

Parasite	Minimum length	Maximum length	Diameter	Other
Females	80mm	100mm	0.24-0.30mm	
Males	40mm	60mm	0.1mm	
Microfilariae	244µM	296µM	7.5-10µM	Sheathed

(Source: Manson-Bahr and Apted, 1984)

2.5.2 Identification of microfilariae

The identification of the microfilaria is dependent, in part, on the detection and identification of microfilarial forms present in patient peripheral blood. Several features are utilized to make the identification (Table 2.1). The presence of a sheath indicates whether the microfilaria is pathogenic (sheathed) or non-pathogenic (unsheathed). The visibility of the sheath is dependent of the type of stain employed. The sheath of *W. bancrofti* stains lightly in Giemsa compared to that of *B. malayi*. Sheaths can be visualized using hematoxylin based stains of thick smears (Manson Bahr and Apted, 1984). The cephalic space is an important feature for identification. *Wuchereria bancrofti* contains a short cephalic space whereas *B. malayi* has a long one. The presence and arrangement of caudal (tail) nuclei is very helpful in the identification of microfilaria. *Wuchereria bancrofti*'s column of caudal nuclei does not extend to the tip of the tail while in *B. malayi* the column of caudal nuclei extend to the tip of the tail; the terminal

two nuclei are separated by a constriction. The sheath stains bright pink with Giemsa stain.

2.5.3 Microfilariae in the body fluids

Microfilariae have been found in a number of body fluids. These include hydrocele fluid (Chandran, *et al*, 2004), blood (Zhong *et al*, 1996), pleural fluid (Abassi, *et al*, 1996), urine, sputum and lymphatic fluid (Lucena, *et al*, 1998). Sputum is matter that is expelled from the respiratory tract, usually a mixture of mucus or phlegm and saliva. It is usually associated with air passages in diseased lungs, bronchi, or upper respiratory tract. Often in cases of chronic cough, sputum can be found to contain blood and is referred to as haemoptysis. When sputum contains pus it is referred to as purulent. A sputum sample is used for microbiological investigations of respiratory infections, which includes pneumoniae and cases of tuberculosis. The best sputum samples contain very little saliva. Saliva contaminates the sample with numerous microbes found in the mouth, which may obscure the situation in the lungs.

There is a presumption that microfilariae gather in the capillaries and other vessels of the lung during their absence from the peripheral blood by the power of agglutination and thigmotaxis (Manson Bahr and Apted, 1984). By injecting heparin, an anticoagulant, at day-time intravenously, microfilariae are released into the peripheral blood for a short period. This makes it possible to diagnose nocturnal *W. bancrofti* during the day. Similarly, the suspected patient can be given a single oral dose of 50–100 mg of diethylcarbamazine (DEC) followed by taking a blood sample 30–45 minutes later (Manson-Bahr and Wijers, 1972, Sherchad, *et al*, 2003). The procedure is said to "flush

out" microfilaria into the peripheral blood during day time, thus making it possible to do a microscopic study during the day. Though sensitivity of microscopy is low, the specificity of this method is comparable to that of night blood surveys (Sasa, *et al*, 1963).

2.6 Clinical manifestation of lymphatic filariasis

Lymphatic filariasis has a wide spectrum of clinical manifestations, most of which are asymptomatic (Beaver, 1970). Symptomatic manifestations of the disease include elephantiasis, hydrocoele, penile, vaginal and breast filariasis (Barry and Marquardt, 1996). The inflammatory responses lead to both lymphoedema found in the limbs, and hydrocoele formation in the testicles (Ash, 1997). During the development of the 'non-inflammatory pathology' the immune system keeps itself 'down-regulated' through the production of contra-inflammatory immune molecules; specifically, the characteristic mediators of Th2-type T-cell responses (IL-4, IL-5, IL-10) and antibodies of the IgG4 (non-complement-fixing) subclass that serve as "blocking antibodies" (Marsden, 1977; Harinath, 1984).

Immune-mediated pathology in lymphatic filariasis most commonly derives from the lymphatic obstructive consequences of the responses to dead or dying worms in the lymphatics (Manson-Bahr and Apted, 1984).

In Tropical Pulmonary Eosinophilia (TPE) there is an immunologic hyper-responsiveness where IgE and other pro-inflammatory molecules directed against microfilariae results in massive hyper-eosinophilia, allergic and other immunologic responses to microfilaria. These reactions cause severe pulmonary functional compromise and tissue destruction that leads to a permanent lung disease (Ash, 1997).

Pathology of this infection is limited to the lymphatics (Ottesen, 1990). There is damage to the lymphatic vessels, caused by an immune response to adult worms as well as a direct action of the parasite, or products released by the parasites (Smith, 2004). Clinical manifestations can be classified as asymptomatic amicrofilaraemic phase, symptomatic microfilaraemic phase, acute phase and chronic phase. This is characterized by the patients not showing any clinical manifestation or microfilaraemia despite exposure to infective larvae (Ottesen, *et al*, 1999). Many of the laboratory diagnostic techniques such as microscopy and antigen-antibody reactions are not able to determine whether patients are infected or not. There is, however, irritation along the lymphatic vessels and a general increase of eosinophils.

Most of lymphatic filariasis patients remain asymptomatic for many months and years, though they have large numbers of circulating microfilariae (Ottesen, 1990). These patients are an important source of infection. Some of these individuals have clinically silent renal abnormalities presenting as microscopic haematuria or proteinuria. Ultrasonography and lymphoscintigraphic imaging have shown that such individuals have dilated and compromised lymphatic functions in the scrotal or lymphatics where the adult worm resides (Freedman *et al.*, 1994; Noroes *et al.*, 1996).

Acute Adenolymphangitis (ADL) consist of intermediate episodes of lymphagitis, adenolymphangitis, funiculitis or epididymo-orchitis with fever. These fever episodes occur once or twice a year, although they may occur more frequently in some patients

(Ramaiah, *et al.*, 1999). During initial months and years, there are recurrent episodes of acute inflammation in the lymph vessel or node of the limb and scrotum that are related to bacterial and fungal super-infections of the tissues with already compromised lymphatic function. These are termed as filarial fevers or Acute dermatolymphangioadenitis (ADLA) which start peripherally (Dreyer and Piessens, 2000). These attacks are associated with transmission intensity (Gyapong, 1998) and host immune response to incoming third stage larvae.

Acute filarial lymphangitis (AFL) is another type of filarial fever in which the inflammation starts in the lymph node with extension down the lymphatic tract. The inflammation appears to be immune-mediated in response to the death of adult filarial worm spontaneously, or as a result of treatment with macrofilaricidal drug. Acute filarial lymphangitis is accompanied by fever, headache, malaise and cold oedema. It is common in scrotal area due to high prevalence of living adult *W. bancrofti* in the lymphatics of the spermatic cord (Noroës, *et al.*, 1996; Dreyer, *et al.*, 1996 c)

The chronic phase is characterized by hydrocele, chyluria (lymph in urine), and elephantiasis (Ottesen, 1990). Elephantiasis affects the limbs and causes disabling and disfiguring (chronic lymphodema) of the limbs, breasts and genitalia, accompanied by marked thickening of the skin. Hydrocoele, a condition which affects the male genitalia is fluid filled balloon-like enlargement of the sacs around the testicles. Hydrocoele is found only with *W. bancrofti* infections and is the most common clinical manifestation of LF (Wijers, 1977). It is uncommon in childhood but it is seen more frequently in post-

puberty, with a progressive increase in prevalence with age (Estambale,*et al.*, 1994). In many endemic communities 40-60% of all adult males have hydrocoele (Wijers, 1977). Chyluria, another form of chronic filarial syndromes is caused by the intermittent discharge of intestinal lymph (chyle) into the renal pelvis and subsequently into urine. The prevalence of chyluria in most endemic areas is very low. Those who develop the chronic form of elephantiasis are usually amicrofilaraemic but have adult worms. Occult filariasis is as a result of hyper responsiveness to filarial antigens derived from microfilaria stages of *W. bancrofti*. Microfilariae will be absent in classical clinical manifestation, but dead or dying microfilariae have been demonstrated in liver, lungs and lymph node biopsies (Webb,*et al.*, 1960). Patients present with paroxysmal cough and wheezing, low grade fever, scanty sputum with occasional haemoptysis, adenopathy and increased eosinophilia. It affects males twice as often as females, and is rarely seen in children.

Bacteria of the genus *Wolbachia* have been associated with filarial nematodes and other arthropods. According to Casiraghi, *et al.*, (2004) *Wolbachia* species, especially *W. pipientis* has been shown to play an important role in the biology of the host and in the immuno-pathology of filariasis (Koneman, *et al.*, 1997). Several filarial species, including, *Wuchereriabancrofti* have been shown to harbor these bacteria, the most common being *Wolbachia pipientis*. Presence of these bacteria in *W. bancrofti* parasites as a symbioant, has made some scientists (Hoerauf, *et al.* 2000) to target antibiotics as possible additional treatment regime. Doxycycline hyclate at a dose of 100mg twice daily for six to eight days was found to eliminate microfilariae from the body.

2.7 Diagnosis of lymphatic filariasis

2.7.1 General physical symptoms

Clinical diagnosis relies mostly on the physical symptoms seen on the patients and the clinical history. Symptoms are however found after a long period of infection, making clinical diagnosis difficult in many of the lymphatic filariasis cases. In the later stages of the lymphatic filariasis, approximately one third of infected individuals present with overt clinical manifestations that may make clinical diagnosis easier. These symptoms include lymphoedema and elephantiasis of the limbs or genitals. In males, the swelling of the testicles is referred to as hydrocoele. Women patients may portray swellings on vulva and breasts. Genital lymphoma may affect 10-50% of the lymphatic filariasis patients (WHO, 2000). The elephantiasis involves the swelling of legs and hands due to obstruction of lymph in the vessels by the parasites. The swelling however has to be differentiated from podoconiosis, a swelling of lower leg (below knee) mostly due to imbalance of body minerals experienced in the East African highlands of Kenya and Ethiopia (Manson Bahr and Apted, 1984,).

Generally, infection is acquired early in childhood; the disease may take years to manifest itself. Indeed, many people never acquire outward clinical manifestations of their infections. Even though there may be no clinical symptoms, studies have now disclosed that such victims, outwardly healthy, actually have hidden lymphatic pathology and kidney damage as well. The asymptomatic form of infection is most often characterized by the presence in the blood of thousands or millions of larval parasites (microfilariae) and adult worms located in the lymphatic system (Jeffrey, *et al*, 1991). Acute episodes of local inflammation involving skin, lymph nodes and lymphatic vessels often accompany the chronic lymphoidema or elephantiasis. Some of these are

caused by the body's immune response to the parasite, but most are the result of bacterial infection of skin where normal defenses have been partially lost due to underlying lymphatic damage. In endemic areas, chronic and acute manifestations of filariasis tend to develop more often and sooner in refugees or newcomers than in local populations continually exposed to infection. Lymphoedema may develop within six months and elephantiasis as quickly as a year after arrival.

Other chronic conditions of the disease include chyluria, pneumonitis, or recurrent infections associated with damaged lymphatics. At the pre-clinical stage of infection clinical diagnosis is usually supported by eosinophilia or intradermal test where overt symptoms are not obvious (Wright and Baird, 1974). Lymphatic filariasis is caused primarily by adult worms living in the lymphatic vessels. Microfilariae released by the female worms circulate in the peripheral blood and are not harmful, but can be ingested by mosquitoes, which transmit the infection from person to person. According to WHO facts sheet 102 (2000), clinical diagnosis of lymphatic filariasis has been extremely difficult making correct diagnosis of lymphatic filariasis to rely on the laboratory findings.

Recently, ultrasonography using a 7.5 or 10 MHz probe has helped to locate and visualize the movements of living adult filarial worms of *W. bancrofti* in the scrotal lymphatics of asymptomatic males with microfilaraemia. The constant thrashing movement of the adult worms in their 'nests' in the scrotal lymphatics is described as the 'filaria dance sign' (Anitha, *et al*, 2001). The lymphatic vessels lodging the parasite are dilated and this dilation is not seen to revert to normal even after the worms are killed by administration of diethylcarbamazine. Ultrasound has been used to study the effect of

drugs on the adult worms and to retrieve them surgically from the dilated scrotal lymphatics. Ultrasonography is not useful in patients with filarial lymphoedema because living adult worms are generally not present at this stage of the disease. Similarly ultrasonography has not helped in locating the adult worms of *B. malayi* in the scrotal lymphatics since the worms are hardly found in the genitalia (Shenoy *et al*, 2000).

The structure and function of the lymphatics of the involved limb can be assessed by lymphoscintigraphy. After injecting radiolabelled albumin or dextran in the web space of the toes, the structural changes are imaged using a gamma camera. Lymphatic dilatation, dermal back flow and obstruction can be directly demonstrated in the oedematous limbs by this method. Lymphoscintigraphy has shown that even in the early, clinically asymptomatic stage of the disease, there are lymphatic abnormalities in the affected limbs of people harboring microfilaria (Freedman *et al*, 1994)

The *Wuchereria bancrofti* genome sequencing was completed in the year 2005. It has three billion base pairs. The genome has some long DNA repeated random segments, one of which has been labeled LDR1 with 1675 bases (Table 2.2) which have been exploited for diagnosis and other research work. In this study 188bp repeated tandem (Table 2.3), labeled as SSP1(Williams *et al*, 2002) was used as the target and was used to design the primers NV-1 and NV-2 (Zhong *et al*, 1996) for the PCR protocols.

2.7.2 Laboratory diagnosis of lymphatic filariasis

Wuchereria bancrofti infections have been diagnosed principally by direct demonstration of the parasite, initially through microscopic detection of microfilariae (mf) in the blood of infected persons (McMahon *et al.*, 1979) and more recently by detection of parasite antigen or DNA. Microscopic detection of parasites may continue to play an important role for demonstration of the impact for community-wide interventions, but measurement of microfilaremia is not an ideal tool for program monitoring or surveillance, both because of the need to examine nocturnal blood to find the parasites in most areas of the world and the relative insensitivity of the commonly used methods for microfilaria detection. Although not detecting the parasite directly, in principle, antibody assays provide sensitive and relatively inexpensive tools to measure filarial exposure; however, the application of antibody tests to precise program monitoring still needs further development. Even as antibody assays are developed and validated, especially for use as potential surveillance tools to detect exposure to infection, there are a number of important research questions that should be answered (Wamae, *et al.*, 1994). Most current assays have focused on detecting antifilarial IgG4 (Rahmah, *et al.*, 2001a) because of the greater specificity of the IgG4 (vis a vis IgG1) response or because of the lack of an IgG4 response to the fusion partner used to express the recombinant antigen. In principle, IgG1 responses should develop sooner after exposure than IgG4 responses; however, it is not clear whether this difference is of practical significance in the field.

Table 2.2: The long DNA repeat 1(LDR1)

```

1      ACTTTTATTT CTGTTTTTTT TTTGTTTTTA TAATTTATGT TTTTATTCTT
51     ATTGTTTGAA TATAGTTTTT TTATTTAGAT GTTATTCGTT TTGTTTAAAT
101    AAATTTTTTTT TTGGTTAATT TTTTAATTAA TTTGTTATTT TTATTTTCAGT
151    AATTTTTTCT TTTTGTATAT TTTTGGTTTC TAATTTTATT TTTGAACTAG
201    ATTTGTTTAT TAACAACCTA GGTTTTTTTT TAAAGTTGTC TTCTGCTCTA
251    TGATTTTTTTT AAATGGCAGC CTTAGCGTGA TGGCATCAAA GTAGCGTAAG
301    TGAATTGTTT TTTAATATTT TCAAGTATGA ATGGAATTTT TAGCAATTTT
351    TTtGTTTATA TTTTATTtTG AATTATTTTT TTTTTTTGTT GGCTTGGTAT
401    AACCTTATTT TTTAATCTTT TTTAATTTTT TTAGTTTTTT TGTTGTCTTA
451    TGGTAAGTGA GCTTATGCTT GTGTTTTAGT AATTTTTTTA GTTTTATTTT
501    TTGTAACCTA AAATTGAAAT TTTAATATT TTATAATAAT GAGAAAAAAT
551    CTTTGCCGGA AATTGGAAGA TCATATCGAt TGTCAGGCAC AATACTATAT
601    AAATTCATAA TTCGTTTCTA ATTCAATTAA AAATCAGTTT TGCTGCCATT
651    AAAGCACCTT AATTATAAAA AATTCATTGT CCAAACCTAAT TGGAAATGAT
701    TACAATTTCT AGGGGTTTTT GTTAATTAAA CAATGAAATC ACTATCATCA
751    ATATGCTTAA TTTCATAATT ATCGTTTGAT TTAGGTGAAT AAAATTTTAT
801    TTTATCATTT TTTTACCATT TTATCATTTA AAATTTGAAA TTTCAAGTCA
851    TTTATAATAA ATTTATACAA AATTTTTTGT AGGTGCAGTG AATTGTTTAA
901    TAGTTTTTTT TAATAGAAAT TAATTAATTA AAACAAAAAA AGCATGAATT
951    AAAAAAAAAT GAATTTCTTA AAAATGAAAC ATTCAAATTT TTTTTCAGT
1001   TCGATTAAAA TTTAGTTGAA TTTATCAATT TCATTATCTA AACTAATTGA
1051   AAATGACATT ATCAAACAGA AATGATGATG ATGCGTTGGA GCCATGTGCA
1101   TATGCAAATA AAAAAGTTTT TTTAAGTGA TTTGCTATTG TTTAATAGTT
1151   TTTTTTTAAT AGAACTTAAC TAATTGGAAC AGAAAAAAAA AAaCATGATT
1201   TGAAAAAAAA GAATTTCTTA AAAAAAGAAA CACTTAAAAA TTTCAAATTT
1251   TTTTGATTAA AATTCAATTA AATTTATCAA TTTCATTATC TGAATTCAAT
1301   TTTATTATTT AAATTAATAA AAATTTTCATT ATTTAAAATT AATGCCTTTT
1351   AGTCATTTTT GATGGTGTAT AATAGCAGCA AACTGATCT TTAATTGAAT
1401   TAGAAATGAA TTATGAATTT ATTTAGTATT TTGATCATCT GGAACGTTA
1451   ATATATCTGC CCATAGAAAT AACTACGGTG GATCTCTGGT TACTACTCTG
1501   AATGGATTAG ACAGTCGACG GAGAAATAAA TGACATATAC ATATTTGTTA
1551   GAGTTATCGA TTCAATTTCT GTGCTGAATT TTTGTGGATT GGTGACGACA
1601   ACTAGGTAA GACTGCTTAC AATTAGTTTG GATAATAAAA TTGACAGATT
1651   TAAGGTGCTT GAATAGTGGC AAAG

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Table 2.3: Tandem repeat sequence SSP1

tttggatg ggtgataata gcagcaaaaa ctgatcttta attgaattag
 aaaactac cacatattat cgtcgtttt gactagaaat taacttaac
 aatgaatta tgaattatt atagtattt gatcatcatctgg gaacgtaat
 ttacttaat acttaataa taccataaaa ctgtagacc ctgcaatta

atatctgcc atagaaataa ctacggatct ctggttatca ctctgaaatg
 tatagacggg tatctttatt gatgcctaga gaccaatagt gagactttac
 gattagacag acagtcgacg gagaaataa tgacatatac atatttgta
 ctaactgtc tgcagctgc ctctttatt actgtatag tataaacaat

gagttatcga tcaatttct gtgctgaatt tttgtggatt ggtgacgaca
 ctcaatagct aagttaaga cagacttaa aaacacctaa ccactgctgt
 actagg

Assessment of antigenemia offers the convenience of any-time-of-day testing and greater sensitivity than testing for microfilariae. Adult *W. bancrofti* release antigens that can be detected in human blood, plasma or serum by immunoassay.

Unlike mf, circulating antigen can be detected with blood collected during the day or night. There are currently two antigen detection tests available. An enzyme-linked immunosorbent assay (ELISA) which detects Og4C3 antigen is highly sensitive and specific; however, this test requires a back-up laboratory infrastructure and is mainly used in research projects (More and Copeman, 1990).

The other antigen test is the ICT filariasis test, which detects AD12 antigen (Weil *et al*, 1997). This test can directly use blood, serum or plasma in the field and provide results in 10 minutes. It is widely used around the world to identify and map endemic areas for inclusion in MDA programs. The test however, remains positive after treatment with DEC/ albendazole or DEC/ivermectin (Pani *et al*, 2000). This is probably because these

drug regimens are not completely effective in killing adult worms, but it is also possible that even when all adult worms are killed, antigen clearance from the blood takes some period of time. Antigens isolated from filarial worms frequently cross-react with antigens from other nematode parasites (Williams *et al*, 2002). However, two recombinant-antigen (Bm14 and Bm-R1)-based antibody tests have been shown to be sensitive and specific for LF infection/exposure. The Bm14 antigen is equally sensitive for either *Wuchereria* and *Brugia* infection or exposure (Rahmah, *et al*, 2001b). The Bm 14 antigen has some cross-reactivity with sera from patients with other filarial infections (loiasis and onchocerciasis), but not with sera from people with non-filarial nematode infections (Ramzy *et al*, 1995). Field studies in Egypt showed that prevalence rates of antibody to Bm14 prior to initiation of MDA were much higher than antigen or mf prevalence rates in young children. In addition, follow-up studies have shown that antibody prevalence rates in children decreased rapidly in the years following implementation of MDA (Gao *et al*, 1994; Rodriguez-Perez, *et al*, 1999). The Bm-R1 antigen performs well in antibody tests for *B. malayi* infection/exposure, but it has limited sensitivity for *W. bancrofti* infection. The Bm-R1 antibody test which is available commercially, detects IgG4 antibodies that is not always associated with LF infection.

Immunochromatographic test, (ICT) is a card test that is used in many of the lymphatic filariasis elimination projects. Although it has some flaws such as insensitivity in low microfilariaemia and remaining positive after treatment (Schuetz, 2000), it was recommended as a diagnostic test for lymphatic filariasis by the GPELF. This is because of its short incubation period and its suitability in using both night and day blood in case of nocturnal periodic filarial infections. In this study, ICT was used as reference test,

where, each blood sample obtained from each patient was tested for comparison with PCR.

Filarial parasites contain a repeated DNA sequence that can be amplified and detected in humans or vectors by PCR. Currently, PCR assays exist for screening blood samples and vectors for *W.bancrofti*. The sensitivity and specificity of the PCR have also made these tests particularly useful for validating the results of conventional parasitologic tests where doubts about species identification arise (Lizotte, *et al*, 1994; Zhong *et al.*, 1996; Hoti, *et al*, 2001a). Although PCR assays are widely used, their utility could be enhanced by improving the availability of standard kits for blood-sample collection, DNA isolation, PCR amplification, and DNA product detection. For use in the widest range of field settings, kits that permit isothermal amplification and visual detection of PCR products without instrumentation would be ideal as well as developing a multiplex PCR that could be used to detect and differentiate *Brugia spp.* and *Wuchereria* from other filarial parasites (Notomi *et al.*, 2000).

The PCR assays are much more sensitive than traditional dissection and microscopy for detecting filarial parasites in mosquitoes (Toure, *et al*, 1998; Cox-Singh, *et al*, 2000; Williams *et al*, 2002). Studies in a number of settings have shown that the percentage of PCR-positive mosquito pools decreased dramatically following MDA. However, a number of practical challenges must be overcome before the PCR can be used with entomologic techniques for xenomonitoring the program effects on LF transmission; particularly. Since different vector species are responsible for transmission of LF around the world, appropriate techniques for trapping mosquitoes to sample the mosquito

populations accurately must be developed. Current PCR tests require a relatively sophisticated laboratory infrastructure and the reagents are expensive. Other PCR assays employing body fluids such as sputum (Abassi *et al.*, 1998) and urine (Lucena *et al.*, 1996) are available though not widely used.

A Dot blot (or Slot blot) is a technique in molecular biology, used to detect biomolecules (Abassi, *et al.*, 1999). It represents a simplification of the northern blot and southern blot or western blot methods. In a dot blot the biomolecules to be detected are not first separated by electrophoresis. Instead, a mixture containing the molecule to be detected is applied directly on a membrane as a dot. This is then followed by detection by either nucleotide probes or antibodies. The technique offers significant savings in time, as chromatography or gel electrophoresis and the complex blotting procedures for the gel are not required. However, it offers no information on the size of the target biomolecules. Furthermore, if two molecules of different sizes are detected, they will still appear as a single dot. Dot blots therefore can only confirm the presence or absence of a biomolecules or biomolecules which can be detected by the DNA probes or the antibody. A radioactive sample can be hybridized to it allowing the researcher to detect variation between samples. The DNA is quantified and equal amounts are aliquoted into tubes in excess of the number of its targets in the samples, such as 10µg for a plasmid and 1µg for a PCR amplicon. These are denatured (NaOH and 95°C) and added to the wells where a vacuum sucks the water (with NaOH and NH₄OAc) from underneath the membrane (nylon or nitrocellulose).

Loop-mediated isothermal amplification(LAMP) is a novel method that is being researched on for diagnosis of many infections, (Notomi *et al*, 2000) including lymphatic filariasis. The method amplifies DNA with high specificity, efficiency and rapidity under isothermal conditions. This method employs a DNA polymerase and a set of four specially designed primers that recognize a total of six distinct sequences on the target DNA. An inner primer containing sequences of the sense and antisense strands of the target DNA initiates LAMP. The following strand displacement DNA synthesis primed by an outer primer releases a single-stranded DNA. This serves as template for DNA synthesis primed by the second inner and outer primers that hybridize to the other end of the target, which produces a stem-loop DNA structure. In subsequent LAMP cycling one inner primer hybridizes to the loop on the product and initiates displacement DNA synthesis, yielding the original stem-loop DNA and a new stem-loop DNA with a stem twice as long. The cycling reaction continues with accumulation of 10^9 copies of target in less than an hour. The final products are stem-loop DNAs with several inverted repeats of the target and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target in the same strand. Because LAMP recognizes the target by six distinct sequences initially and by four distinct sequences afterwards, it is expected to amplify the target sequence with high selectivity. This is logistically challenging in cases of mass diagnosis, besides non-compliance by the communities involved, as the people are sensitive to being bled (Lucena *et al.*, 1998).

The antigen test recommended by WHO uses day blood (Weil *et al.*, 1997) but it has shortcomings such as insensitivity in cases of low microfilarimae (Pani *et al.*, 2000, Schuetz *et al.*, 2000) and may not be useful for surveillance in the post elimination phase.

2.8 Treatment of lymphatic filariasis

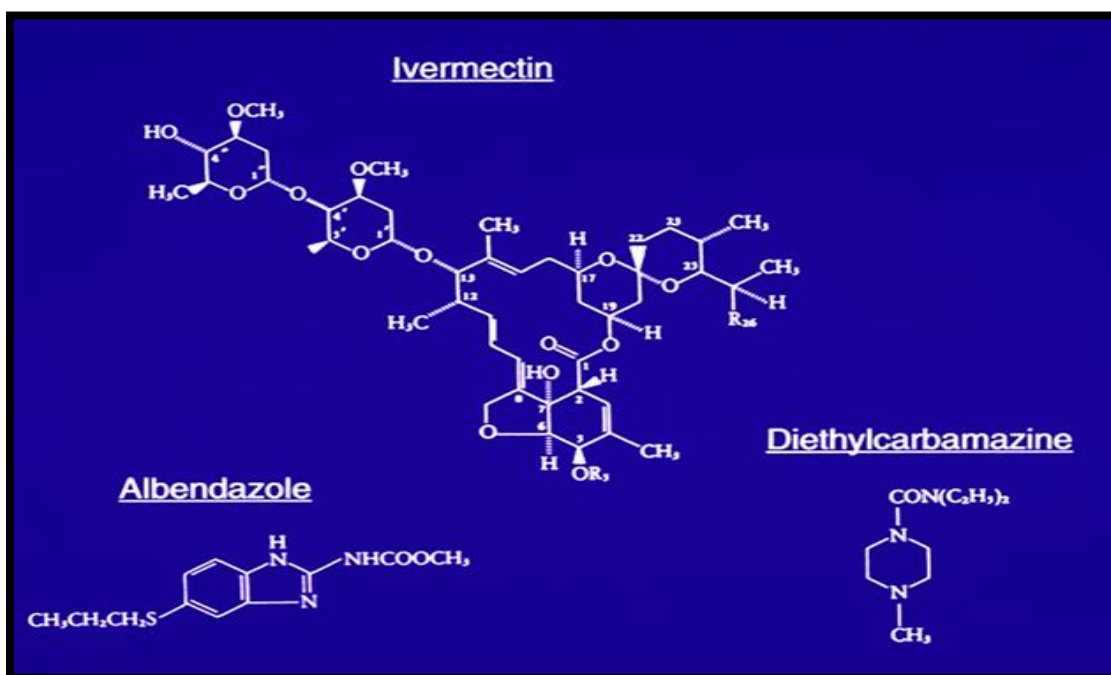
Diethylcarbamazine (DEC) is an effective drug against both microfilaria and adult worms (Figure 2.5). It lowers the blood microfilaria levels markedly even in single annual doses of 6 mg/kg, and this effect is sustained even at the end of one year. Diethylcarbamazine is the drug of choice for the treatment of lymphatic filariasis (WHO, 2000). Even though DEC kills the adult worms, this effect is seen in only 50% of patients. By ultrasonography (Amaral, *et al*, 1994) it is shown that even single doses of DEC kills the adult worms when they are sensitive to the drug. When they are not sensitive even repeated doses do not have any effect on the adult parasite (Noroes *et al*, 1997). This drug does not act directly on the parasite but its action is mediated through the host immune system. The earlier recommended dose of this drug was 6 mg/kg given daily for 12 days. Recent studies have however shown that single dose of DEC 6mg/kg is as effective as the above standard dose given for 12 days (Ottesen *et al*, 1999). The sustained destruction of microfilaria by this drug even in annual single doses makes it a good tool to prevent the transmission of lymphatic filariasis. The adverse effects produced by the drug are seen mostly in patients who have microfilaria in their blood and are due to their rapid destruction which is characterized by fever, headache, myalgia, sore throat or cough lasting for 24 to 48 hours. Reactions are usually mild and self-limiting requiring only symptomatic treatment. Recent trials have clearly shown that DEC has no action either in the treatment or prevention of the acute ADL attacks occurring in lymphoedema (Anitha *et al*, 2001). DEC is the drug of choice in the treatment of Tropical Eosinophilia syndrome where it is to be given for longer periods of 3 to 4 weeks.

Ivermectin acts directly on the microfilaria and in single doses of 200 to 400ugm/kg keeps the blood microfilaria counts at very low levels even at the end of one year, like DEC. The adverse effects noticed in microfilaraemic patients are similar to those produced by DEC but are milder due to the slower clearance of the parasitaemia. Ivermectin (Figure 2.5) has no proven action against the adult parasite or in tropical eosinophilia (Ottesen *et al*, 1990). Ivermectin is the drug of choice for the treatment of onchocerciasis because of its safety and efficacy, when compared to DEC. It is also the drug of choice for prevention of filariasis in African countries endemic for *Onchocerca* and *Loa loa*, where DEC cannot be used due to possible severe adverse reactions (Thomson, *et al*, 2000).

Albendazole is an anthelmintic drug known to destroy the adult filarial worms when given in doses of 400mg twice daily for two weeks. The death of the adult worm induces severe scrotal reactions in bancroftian filariasis since this is the common site where they are lodged (Schwartz, *et al*, 2009). Albendazole has no direct action against the microfilaria and does not immediately lower the microfilaria counts (Addiss, *et al.*, 1997). But when given in single dose of 400 mg in combination with DEC or ivermectin, the destruction of microfilaria by these drugs becomes more pronounced. Albendazole (figure 2.5) combined with DEC or ivermectin is recommended in the global filariasis elimination program. The strategy that appears most suitable for elimination of filariasis in India is the administration of single annual dose of albendazole 400mg along with DEC 6 mg/kg body weight. This not only will prevent transmission of filariasis in the

community by reducing the microfilaria levels, but also has the added benefit of clearing the intestinal helminthes (Bockarie *et al*, 2002).

The most distressing aspect of lymphatic filariasis is the acute attacks of Acute Adenolymphangitis, ADL, which prevent the patient from attending his daily activities. This results in considerable economic loss and deterioration of quality of life of the affected population. Prompt treatment and prevention of ADL are of paramount importance. Bed rest and symptomatic treatment with simple drugs like paracetamol are enough in mild cases. Any local precipitating factor like injury and bacterial or fungal infection should be treated with local antibiotic or antifungal agents. Moderate or severe attacks of ADL should be treated with oral or parenteral administration of antibiotics depending on the general condition of the patient.



Source: Bockarie. 2002

Figure: 2.6: Medicines used for treatment of lymphatic filariasis

Since the ADL result from secondary bacterial infections, systemic antibiotics like penicillin, ampicillin or cotrimoxazole may be given in adequate doses till the infection subsides. Bacteriological examination of swabs from the entry lesions may help in selecting the proper antibiotic in severe cases. Many recent studies have shown that with proper 'local care' of the affected limb these ADL attacks can be prevented even in case of severe lymphoedema. This 'foot-care program involves; washing of the affected area, especially the webs of the toes and deep folds of skin, with soap and water twice a day and wiping dry with a clean cloth to avoid moisture retention. Preventing or promptly treating any local injuries or infections using antibiotic and antifungal ointments. This exercise should be done on a daily basis, and where possible twice in a day. The patient should wear properly fitting shoes regularly and keep the affected limb elevated especially at night to reduce the swelling. In patients with late stages of oedema satisfactory local care of the limb is not possible due to deep folds of skin or warty outgrowth. To prevent repeated ADLs in such patients, long term antibiotic therapy using oral penicillin or long acting parenteral benzathine penicillin is indicated (Shenoy, *et al*, 1998)

There are various surgical options like lymph nodo-venous shunts, omentoplasty, excisional surgery and skin grafting (Danda, *et al.*, 1985). Even after surgery the care of the limb should be continued for life, to prevent recurrence of the swelling (Appendix vii). Prolonged treatment with oral or topical coumarin or flavonoids is said to be beneficial in reducing the lymphoedema, by stimulating the macrophages to remove excess proteins from oedema fluid. Rarely coumarin is reported to produce idiosyncratic hepatitis (Manson-Bahr and Apted, 1984).

2.9 Control and prevention of lymphatic filariasis

Early treatment with this Dicarbamazine in a patient having microfilaria in the blood may destroy the adult worms and logically prevent the later development of lymphoedema. Equally important is the prevention of ADL attacks in patients with underlying dysfunction since the occurrence of lymphoedema and its progression are due to repeated infections. Once lymphoedema is established there can be no cure. Below are some treatment modalities which may offer relief and may prevent further progression of the swelling: These include use of elastocrepe bandage or tailor made stockings. Keeping the limb elevated at night or while resting, after removal of the bandage. Exercising and massaging of the affected limb by the patient. The suggested treatments tend to stimulate the lymphatics and to promote flow of lymph towards larger patent vessels. The use of intermittent pneumatic compression of the affected limb using single or multicell jackets plus heat therapy either using wet heat or hot oven may further reduce the progression of the swelling.

World Health Organization (WHO) initiated a Global Program for Elimination of Lymphatic Filariasis, (GPELF) by mass treatment of the infected and those-at-risk, estimated at 1.1 billion (WHO, 1998, Figueroa, 1999). Many of the lymphatic filariasis endemic countries have started their national programs including Kenya which started in 2003 (Njenga *et al*, 2004). Vector control for LF, focusing on indoor residual spraying and the use of long lasting insecticide treated nets (LLITNs) overlaps with vector control strategies for other diseases such as malaria. In countries with both malaria and LF, particularly in Africa, integrated strategies for vector management are poised to benefit

control programs for both diseases. There is currently no vaccine for the prevention of lymphatic filariasis.

2.10 Challenges in mass diagnosis of lymphatic filariasis

Diagnostic tools are essential at each step of the lymphatic filariasis elimination; initially for defining areas in need of mass drugs administration through mapping activities, then for monitoring the progress of programs following implementation of MDA, and finally for verifying the absence of infection both in areas where MDA has been conducted and in settings where LF was present (WHO, 2007). As the elimination programs approach their planned end points, that are five or more years with greater than 80% MDA coverage, it is necessary to determine whether transmission has been interrupted and whether MDA can be stopped. Parasitologic assessment, requires testing thousands of persons to demonstrate that infection levels are below 0.1%, the level targeted by WHO (2007). Intensive data collection, in the context of the programs, is needed to determine how different tests and vector infection correlate and compare with as a programmatic endpoint. Age- and sex-specific longitudinal data collections (in a variety of epidemiologic settings) are the key to developing the ability to recognize with confidence when transmission has been interrupted. To address these testing the effectiveness of the available diagnostic assays in the application of guidelines is a priority. Since test specificity is of greatest concern for this approach, algorithms used to confirm suspected infection or exposure must be developed and validated. In addition, population movement from endemic to non-endemic regions carries with it the risk of possible introduction of LF transmission. Surveillance methods to address these situations, based on diagnostic and serologic methods, should be evaluated.

In principle, antifilarial antibody responses can serve as markers of filarial exposure and transmission, providing evidence of infection or exposure in an individual before the development of antigenemia or microfilaremia, since antibody responses may develop within weeks to months following exposure to infective larvae (Lammie, *et al*, 2004). This theory has however been compromised, through a recent multicenter evaluation which identified several promising candidate antigens for serologic assays to monitor LF, but none was specific for *W. bancrofti* (Lammie *et al*, 2004). Since LF elimination programs may have limited effect on persons with or exposed to *Mansonella*, *Loa*, or *Onchocerca* volvulus infections, the absence of a specific assay for *W. bancrofti* make it difficult to use current antibody tests for program monitoring in areas where these infections are co-endemic with LF. For example, testing young children in Nigeria for antifilarial antibody using the Bm14 antigen is not useful as a method to detect recent exposure to *W. bancrofti* as an incident antibody response may only represent exposure to other filarial parasites. Therefore, a valuable research goal is the development of an antibody test that is specific for *W. bancrofti* to enhance the usefulness of 'exposure antibody' assays for program monitoring in sub-Saharan Africa.

Collection of blood specimens remains in many places a significant challenge especially in cases of mass diagnosis. Blood poses the risk of infection with blood borne diseases and especially the hepatitis, HIV and AIDS (Marcus, 1988). Trained medical teams are required and non-compliance by the communities involved is evident due to sensitivity to being bled (Lucena *et al.*, 1998). Therefore, to address this practical program concern, attempts should be made to adapt the available assays to work with oral fluids or urine that would provide program managers with other

options to achieve complete or adequate sample collection (WHO, 2007). Antigen testing currently does not detect *Brugia* infections which account for approximately 10% of the world's burden of LF. Antigen test results often remain positive long after treatment with DEC/ albendazole or DEC/ivermectin (Weil *et al*, 1991; Eberhard *et al*, 1997). This is probably because these drug regimens are not completely effective in killing adult worms, but it is also possible that even when all adult worms are killed, antigen clearance from the blood takes some period of time. Furthermore, when antigen prevalence rates are low in young children, as they are in some LF-endemic areas, measuring incidence rather than reductions of antigen prevalence may be more valuable for monitoring changes in transmission of filariasis following implementation of MDA.

Vector control for LF, focusing on indoor residual spraying and the use of long lasting insecticide treated nets (LLITNs) (Poopathi and Rao, 1995), overlaps with vector control strategies for other diseases such as malaria. In countries with both malaria and LF, particularly in Africa, integrated strategies for vector management are poised to benefit control programs for both diseases but this can only be achieved through employment of specific and sensitive testing tools in monitoring and surveillance of the lymphatic filariasis in both the population and the vectors.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Area

The study was carried out in the Tana Delta District, in Kenya's North coast. The area is a wetland complex at an altitude of 0 - 37m (Figure 3.1). This low-lying area borders the Indian ocean near Ungwana (or Formosa) Bay and higher land to the east and west. There are fresh and brackish lakes (such as Shakababo and Bilisa) and streams, with vegetation which includes forests, bush-land, grassland and woodland on the riverbanks (Gachathi, *et al*, 1994; Quentin, 2005). Flooding is common, sometimes twice in a year during the short and long rains. Mangrove forest grows at Kipini in the Tana estuary and along the network of channels further south (Bird life International, 2005). Some of the Lakes maintain true aquatic plants and populations of several species of fish (Britannica, 2009).

Tana River Delta District is named after the 1,014 km long Tana River which begins on the slopes of the volcanic Mounts Kenya and the Aberdares in Central Kenya (Kibunja, 2002). The river is slow and meandering in the lower half of its course to the Indian Ocean. The Tana Delta District includes most of the 64 square Kilometers of East Africa's remaining rain forests and is home to the wildlife that includes two rare primates, the red Colomus and the crested Mangabey monkey (Ng'weno, 2009). The primates form a source of non-human filarial worms (Gutierrez, 1990, Orihel and Eberhard, 1998), which can be transmitted through the mosquitoes (Anderson, 1992). Most people live in small villages next to water sources (Hamerlynck, 2009). Drinking water is obtained from the river, ponds or dugout shallow wells. Abundance of the stagnant water and the warm temperatures makes the environment conducive for breeding mosquitoes which are **vectors** for *W. bancrofti* (Bogh *et al.*, 1998). The delta opens at Kipini town in the Formosa (Ungwana) bay, where there are old settlements that grew as a result of contact between the local inhabitants and the Persian/Arabian cultures dating as far back as 1200 to 1250 AD.



Figure 3.1: Map of the study area

Source: Kenya coast files

The Tana Delta district forms a part of the former Tana River District, which by 1999 census report, had a population of 180,901. It has an area of 38,446 Km² and a population density of 5 people per Km² (Government of Kenya Census Report, 1999). Most of the population is composed of the Pokomo ethnic group, who are mostly farmers using the Tana River water to irrigate their crops. They plant rice and other crops, such

as maize, bananas and sweet potatoes (Wakanene and Njue, 1994). Some Kenyan communities from the western and central regions have settled in this area and are responsible for an active and thriving fishery and horticultural farming, while Orma pastoralists use the wetlands grazing areas for their livestock.

The Swahili are mainly found at Kipini town (Kibunja, 2002). Other cultures, mainly Bantu (Pokomo, Giriama and others) and Cushites (Orma, Wardia and Somali) from the up country have recently inhabited the area. The popular houses (90%) in the villages are made of wood, mud and thatched with palm fronds. A fairly good number may have aggregate stones mixed with mud for walls and rarely roofed in corrugated iron sheets. Due to poverty which stands at >50% (Luke *et al*, 2005), many of the houses are in dire need of repair leaving large gaping holes in the roof and walls. Most houses have only open spaces for doors or windows. This makes it easy for high mosquito human contact.

3.2 Study design and methodology

This study was a cross sectional study of human and mosquito samples. Consenting human participants were examined by a clinician before collecting samples of sputum, urine and serum from each of the participant. Mosquito samples were collected using traps and spraying techniques. The data were first collected in field note books and later transferred to computers, where it was recorded using the excel spreadsheet. Confidentiality of the participants was maintained at all times.

3.3 Selection Criteria

Any willing participant who had lived in the study area for at least five years and was above the age of five was eligible.

3.4 Exclusion criteria

People who had not lived in the study area for the last five years and children below four years of age were excluded from the study.

3.5 Sampling techniques

3.5.1 Sample size determination

Sample size was determined using the formula of Fisher *et al.*, (1998).

$$n = \frac{Z^2 pq}{d^2}$$

Where;

n = minimum sample size required

Z = standard normal deviate from the mean

p = proportion of the study population estimated to have *W. bancrofti* infection (i.e. 0.2)

q = 1 – p

d = the level of statistical significance set at 0.05

Substituting in the above formula,

$$= \frac{1.96^2 \times 0.2 \times 0.8}{0.05^2} = 245.8 \text{ or } 246$$

A minimum number of 246 were required for the study; however, eight hundred and two persons volunteered and were recruited for the study.

3.5.2 Collection of samples

All the samples were used in all tests; microscopy, immunochromatographic test, sputum, serum and urine PCR assays. Each participant was examined by a clinician and asked to give blood, sputum and urine samples (Appendix 1). Approximately 1ml of sputum was collected from each participant into a 15ml tube containing 1ml 0.2M

Ethylene-diamine tetra acetic acid (EDTA). Where applicable, sputum was induced by deep cough following a brief jogging or push-up exercise (Abbasi *et al.*, 1996).

Blood samples for microscopy, ICT and PCR assay consisted of 1000µl of blood bled using needle and syringe with 0.1M EDTA, which served as both anti-coagulant and a DNA preservative. Approximately 50ml of urine was obtained from each donor after advice on how to collect a clean mid-stream sample. The urine was collected in a tube (Corning™), to which 1ml 5M EDTA had been added. All samples were labeled with participant's name, sex, age, date of sample collection and the location.

Mosquitoes were collected using three methods namely; pyrethrum-spray catch, CDC light and gravid traps. A knockdown spray catch protocol described by Chadee *et al.*, (2002) was used to collect mosquitoes. Briefly, after notification to the family, white sheets were spread in all the rooms and house eaves. The household was then sprayed using a pyrethrine based insecticide for 15 minutes. All the knocked down mosquitoes were collected, dried, sorted and stored in cryovials 10/vial (Figure 3.2). The vials were labeled with collection date and number of the household.

Two trapping methods were used as advised by the manufacturer; John W. Hock Company, USA. Briefly, the Centers for Disease Control light traps with a standard 6V 100mA incandescent bulb and powered by 4 dry cell batteries (Appendix 3) were hung 5 to 6 feet above the ground in selected rooms, living room, bedroom and eave from 7:00 pm to 7:00 am. The mosquitoes were then collected and data was recorded for each site, including the number of mosquitoes trapped. Similarly, the CDC gravid traps, designed like the light trap but with additional water basin at the base with a fermented grass

infusion to attract gravid mosquitoes as they come to lay eggs, was set as instructed. The grass infusion was made by adding 0.1 kg of grass to 23 liters of tap water, and allowing the infusion to incubate for 5 days (Service, 1977). The captured mosquitoes were removed early the following morning and recorded appropriately.



Source: J Kagai

Figure 3.2: Sorting mosquitoes from the Tana Delta

3.5.3 Mosquito identification and dissection

Identification of mosquitoes was done visually as well as microscopically using the identification keys (Wharton, 1962; Peyton and Scanlon, 1966; Vinogradova, 2000; WHO, 1975). The various features used during identification included palpi, abdomen, thorax and wing structure. All female mosquitoes were selected for dissection. All equipment used for dissection was first autoclaved to ensure sterility. Mosquitoes were dissected on a glass slide using a set of dissection needles to separate the head, thorax, and abdomen. Each mosquito body segment was dissected separately on the same slide

using a dissecting microscope at X10 magnification. The three body segments were teased apart and examined stereoscopically to reveal microfilariae. Mosquito infection status was noted (Goodman *et al.*, 2003), as well as the larval stage of microfilariae. All body segments belonging to each mosquito were transferred into vials for DNA extraction, PCR and dot blot hybridization.

3.6 Microscopy and Immunochromatographic tests (ICT)

Examination of blood samples by microscopy was performed according to McMahon (1979). Briefly, 100µl of whole blood was added to 900µl of 3% acetic acid. This was then added to the special counting chamber and examined under low power. All microfilariae were counted and the results given as microfilaria counts per ml. In each case the counts were done three times and an average count calculated.

From each of the blood sample obtained, 100µl was applied onto the appropriate spot on the ICT card according to the manufacturer's instructions (Binax Inc. USA). The card was then labeled with sample number and date, incubated at room temperature for 15 minutes and the results read from the cards' window (Weil, *et al.*, 1997). The cards were retained at 4°C for any necessary references.

3.7 DNA EXTRACTION

3.7.1 DNA extraction from sputum

The DNA extraction from sputum was carried out as described by Abbasi *et al.*, (1996) with some modifications. Briefly, DNA was extracted by treating 200µl of sputum with 200µl of 1M NaOH– 1% Triton solution in a microfuge tube. The mixture

was vortexed and incubated at 65°C for 30 minutes. The mixture was heated to 100°C for 5 minutes, cooled quickly on ice and spun briefly at 1500g for one minute to bring down all debris. To precipitate DNA, 625µl or 2-3 times the volume of cold absolute ethanol was added and the samples kept at -80°C overnight. The precipitated DNA was washed three times with 70% ethanol to get rid of extra precipitated salts. The DNA was re-suspended in 50µl Tris-EDTA (TE) buffer, pH 8.0. The DNA was further filtered through Qiagen DNA clean-up kit and stored at -20 °C till needed.

3.7.2 DNA extraction from urine

About 20ml urine was filtered through Nitran N45™ (Fischer *et al.*, 2003). The filters, which are made of nitrocellulose paper, were transferred into 0.5ml eppendorf tubes and elution done using 500µl of NaOH-1%Tris pH 8.0 solution then warmed at 65°C for 60 minutes and neutralized with 10µl of 1M HCl. The samples were then centrifuged for 5 minutes at 14000 rpm and supernatant transferred to a fresh eppendorf tube and boiled at 95-100°C before cooling quickly and incubating at -70°C (Lucena *et al.*, 1996). After washing in 70% ethanol, the pellets were dried in an eppendorf concentrator 5301™ then re-dissolved in 50µl TE. The DNA was further filtered through Qiagen DNA clean-up kit and stored at -20 °C till needed.

3.7.3 DNA extraction from serum

Wuchereria bancrofti DNA from serum or plasma was extracted as described by Zhong *et al.*, (1996) with modifications. Briefly, 50µl serum sample was added into sterile eppendorf tubes and mixed with 198µl of NaOH + 1% triton. This was warmed at 65°C

for 30 minutes in a thermo mixer and pH adjusted to 8 with HCl or NaOH. The mixture was centrifuged at 4°C for 5 minutes before transferring the supernatant to new eppendorf tubes and heating at 100°C for 5 minutes. This was cooled quickly before adding 400-500µl of absolute ethanol. It was incubated at -80°C for 16hrs and spun at 4°C, 14000rpm for 20minutes. The precipitated DNA was washed thrice with 70% alcohol and spun dry in a micro concentrator for 1hr. It was further dried at 65°C for 30minutes to remove all traces of alcohol. The DNA was then dissolved in 50µl TE buffer. The DNA was further filtered through Qiagen DNA clean-up kit and stored at -20 °C till needed.

3.7.4 DNA extraction from mosquitoes

The mosquito DNA was extracted according to the method of Aljanabi and Martinez, (1997) with modifications. Instead of a pool of 10 mosquitoes, a single mosquito was ground in 100µl of grinding buffer in a microfuge tube ensuring no large fragment remains. The ground mosquito was placed into a tube immediately in a water bath at 65 °C for 30 minutes. Fourteen µl of 8 M potassium acetate was added and mixed by vortexing. This was then cooled on ice for 30 minutes before centrifuging at 15000 counts per minute (CPM) for 10 minutes. The supernatant was saved in a new sterile micro vial. Two hundred µl of absolute ethanol was added and incubated at -80 °C for 30 minutes or longer to precipitate the DNA. The solution was spun at 15000 CPM for 20 minutes. The ethanol (supernatant) was then poured off. The DNA was washed with 200µl of 70 % ethanol, before rinsing with 200 µl of absolute ethanol. The DNA was

dried and re-suspended in 50µl Tris-EDTA (TE) buffer, pH 8.0. The DNA was further filtered through Qiagen DNA clean-up kit and stored at -20 °C till needed.

3.8 Polymerase chain reaction

Positive control used was long DNA repeat 1 (LDR1) supplied by Prof. Hamburger, Hebrew University, Israel. Forty five micro-liters of polymerase chain reaction mixture composed of Taq DNA polymerase Buffer (10µl), 10mM of each dNTP (2µl), 5pmol direct primer, 5pmol reverse primer, Phusion DNA polymerase 5µl and 79.5µl of double distilled sterile water, was added to each 10µl of the prepared DNA. For optimal thermocycling, the DNA was denatured at 94°C for 5 minutes, annealed at 54°C for 1minute and elongated at 72°C for 1 minute. This was repeated for 35 cycles, before a further elongation step at 72°C for ten minutes. The primers used were those previously identified by Zhong, *et al.*, (1996) as NV1 5' CGTGATGGCATAAAGTAGCG 3' and (3' CCTCACTTACCATAAGACAAC 5'), and used in PCR assays for mosquitoes (Weil, *et al.*, 1999; Ramzy, 2003) sputum (Abbasi *et al.*, 1996) and urine (Lucena, *et al.*, 1996). These primers amplify a region 188 bp long of *W. bancrofti* DNA sequence.

3.9 Gel electrophoresis

Agarose gels were prepared by dissolving 1.2g Agarose (Nusieve GTG) in 100ml of Tris-sodium acetate-EDTA (TAE) buffer (Sambrook *et al.*, 1989) and heating until dissolved. This was then cooled in a water bath at 46-50°C. Ethidium Bromide at a concentration of 0.5µg per 80-100ml of melted agarose was added. The molten agarose

was carefully poured into a gel tank with the combs set appropriately and let to set for 30 minutes. To 10µl of each sample 2µl of loading buffer comprising of Bromophenol blue, Xylenecyanol FF, Ficoll and distilled water, was added (Sambrook *et al.*, 1989). Enough TAE buffer (150ml) was put into the gel tank to just cover the gel in the electrophoresis tank. The comb was removed from the gel, and 6µl of each diluted sample loaded into the wells. Negative and positive controls and a marker were included in the appropriate wells. The electrophoresis was run at 100mV for 50 minutes and the bands viewed on an UV viewer, (Vilber Lumart™). The gels were photographed using Mini-visionary, Fotodyne equipment for permanent records.

3.10 Ethical consideration

Before the initiation of this study, ethical clearance was obtained through KEMRI Ethical Review Committee. All the participants aged >18 years were registered in a house-to-house survey for the study after giving their informed consent (Appendix 2). The younger participants aged below 18 years had parents or guardians give consent on their behalf to participate in the study. In this younger group, verbal consent from the participants themselves was sought as well.

3.11 Data analysis

The results were recorded along with the sample identification parameters such as name, age, sex, location and date of collection in a Microsoft excel spread-sheet for statistical analysis. Firstly the data was collected in field note books and then transferred into a computer, where it was recorded in Excel Spreadsheet. Using the statistics software SPSS V12, the results were subjected to *chi-test* (χ^2) for prevalence comparisons between

sexes, ages, tests, vectors and human prevalence using the three techniques (Greenhalgh, 1997). Spearman correlation and student *t*-test were used to identify any statistical significance between prevalence in mosquitoes and the human population.

CHAPTER FOUR: RESULTS

4.1 Results from optimization of PCR assays

The results in Figures 4.1 to 4.4 demonstrate 3% agarose gel bands of *W.bancrofti* DNA, obtained by using the modified PCR assays after 30 minutes run at 100mUV on a Consort™ microcomputer electrophoresis system. Positive bands were taken as those corresponding to 188bp on the Fermentas™ M100bp marker. Figure 4.1 shows results obtained when two enzymes, phusion and Taq polymerase were used, phusion demonstrated more visible bands compared with Taq polymerase. For each enzyme in this experiment, duplicate tests of the same sample were used. Upon serial dilution of LDR1, the modified PCR assay was able to detect 0.04pg of *W. bancrofti* DNA (Figure 4.2). Figures 4.3 shows bands produced by sputum, serum and urine samples obtained from one of the infected study participants. In the case of the urine, a stronger band was obtained from DNA extracted from 20mls of the participant's urine sample compared to DNA extracted from 10mls of the same urine sample. Figure 4.4 shows typical bands obtained by various samples of the study participants.

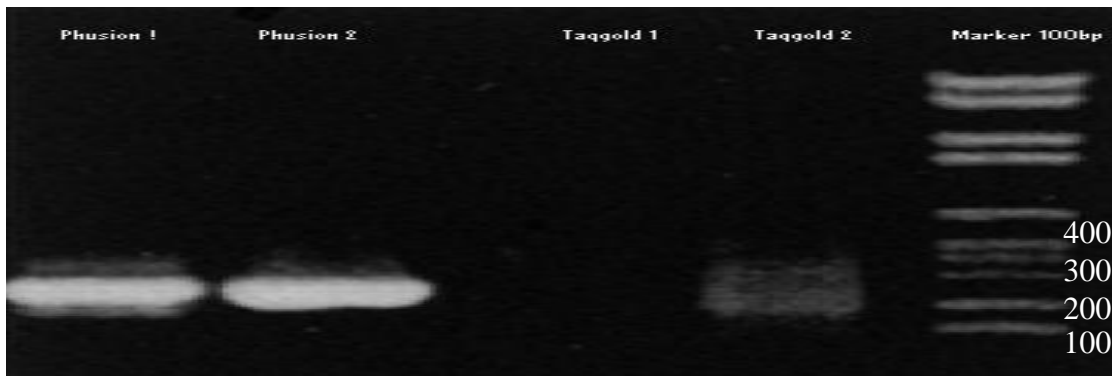


Figure 4.1: Comparative PCR reactions using phusion enzyme and *Taq* polymerase

Figure 4.2: Bands produced by LDR1serial diluted down to 0.04pg.

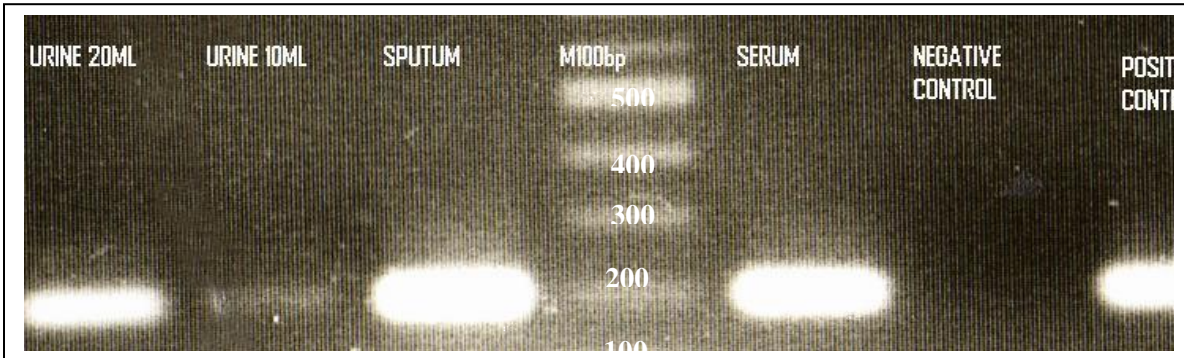
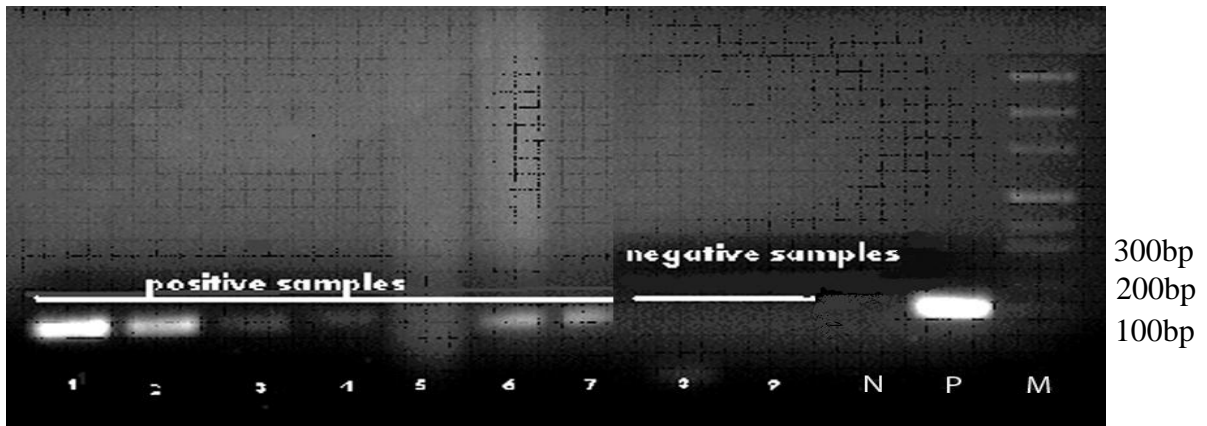


Figure 4.3: Gel bands from urine, sputum, serum, negative and positive controls. A marker 100bp is in the middle for easy comparison



Key: 1-9 =DNA samples; N=negative control; P=positive control; M=marker

Figure 4.4: Typical agarose bands from the study samples

4. 2: Demographic data

From the data collected, the number of males (Table 4.1) in the study group was 58.9%, while the number of females was 41.1%. The total number of participants was 802 individuals. Majority of the study population (Table 4.2) came from Idsowe village

16.1%, Ngao 15.6%, Tarasaa 10.3% and Wema 9.2%. Furaha and Chamwanamuma had 1.9% each, which was the least percentage from the study villages. Other villages included Maziwa (3.6%), Kipini (4.7%), Matomba (6.4%), Odda (6.9%), Dalu (7.4%) and Kilelengwani (7.9%).

The study population ranged from 5 to 82 years (Figure 4.6) in age. There were 114 (14.5% of total participants) children aged below ten years. In this group, 54 were females, making 16.4% of all females in the study population. Similarly, 60 were males, making 12.7% of all males in the study population.

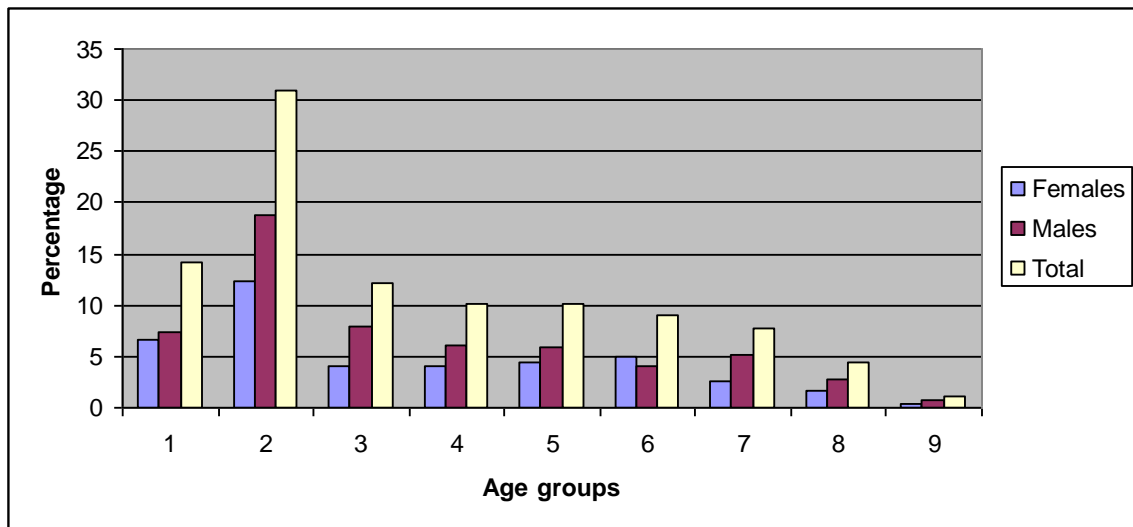
Table 4.1: Distribution of the study population by gender

Gender	Frequency	Percent
Female	330	41.1
Male	472	58.9
Total	802	100

The age group 11-20 years was the majority in the study population at 249 (31%). This group was followed by <10 years group, 21-30 years group (97) and 31-40 years group with 81 (10.1%). The group with least number was >81, which had only 9 (1.1%) people. In this group, six were males accounting for 1.3% of male population and 3 females, accounting for 0.9% of the female population.

Table 4.2: Distribution of the study population by location

Village	Frequency	Percent
Chakamba	19	2.4
Chamwanamuma	15	1.9
Dalu	59	7.4
Furaha	15	1.9
Kilelengwani	63	7.9
Idsowe	129	16.1
Kipini	38	4.7
Matomba	51	6.4
Maziwa	29	3.6
Ngao	125	15.6
Odda	55	6.9
Shirikisho	47	5.9
Tarasaa	83	10.3
Wema	74	9.2
TOTAL	802	100



Key: Age groups in years: 1= <10; 2=11-20; 3=21-30; 4=31-40; 5=41-50; 6=51-60; 7=61-70; 8=71-80; 9=>81

Figure 4.5: Distribution of age groups and gender in the study population

4.3 Mass diagnosis of *W. bancrofti* infections in the study area using clinical symptoms, microscopy, ICT and PCR assays

4.3.1 Clinical diagnosis

Upon registration, the participants were observed for clinical symptoms of lymphatic filariasis (LF). The symptoms included elephantiasis or edema of the limbs (Figure 4.5), hydrocele and chest pains and were recorded as examined in the study (Figure 4.7). People showing edema of legs and hands (elephantiasis) were 8.4% (67/802), hydrocele or swelling of the testicles 3.1% (25/802) and chest pains 1.1% (9/802). Seven hundred and one out of 802, or 87.4% individuals had no symptoms. The total number of individuals with symptoms was 12.6% (101/802).



Source: Donald McNeil

Figure 4.6: Odema of limbs; one of the three symptoms examined in the study

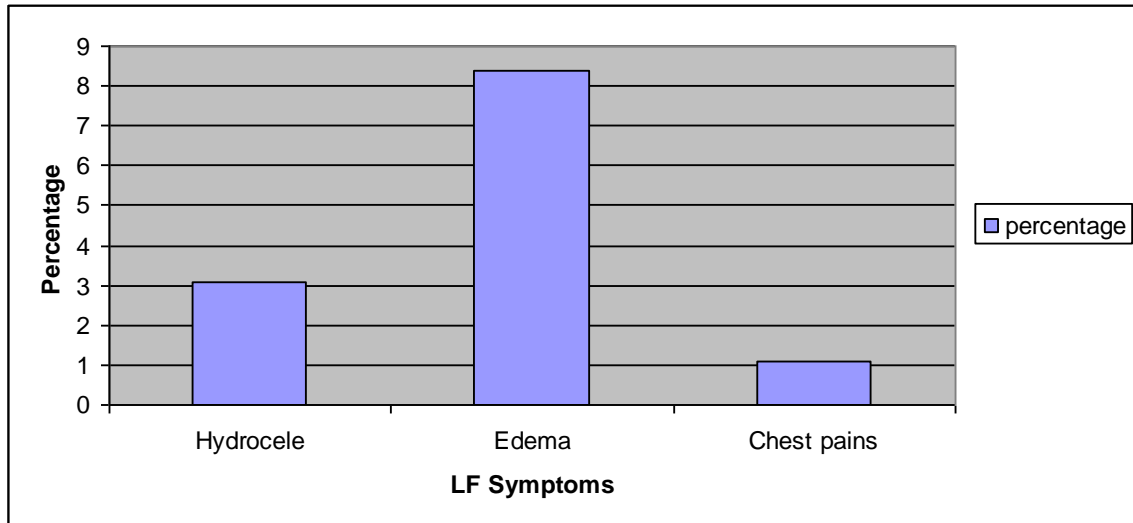


Figure 4.7: Prevalence of lymphatic filariasis symptoms in the Tana Delta

4.3.2 Microscopy and Immunochromatographic test (ICT) results

Microscopic examination of the participants' night blood revealed 43 out of 802 individuals to be microfilarimic. This number represented 5.4% of the study population. While using the WHO recommended rapid test ICT, 51 people or 6.4% of the population were positive (Figure 4.8). The highest prevalence using microscopy was in Chakamba village with 21% (4/19) the lowest prevalence of 0% (0/15) was found in Chamwanamuma. Similarly, for ICT, the highest prevalence was in Chakamba with 26.3% (5/19) and the lowest prevalence at 1.7% (1/59) in Dalu.

4.3.3 PCR assays' results.

When PCR assay utilizing sputum was used, more individuals 9.6% (77/802) were detected as having *W. bancrofti* DNA. Similarly, PCR assay using urine and serum showed 6.7% (54/802) and 6.6% (53/802) *W. bancrofti* DNA positive individuals respectively.

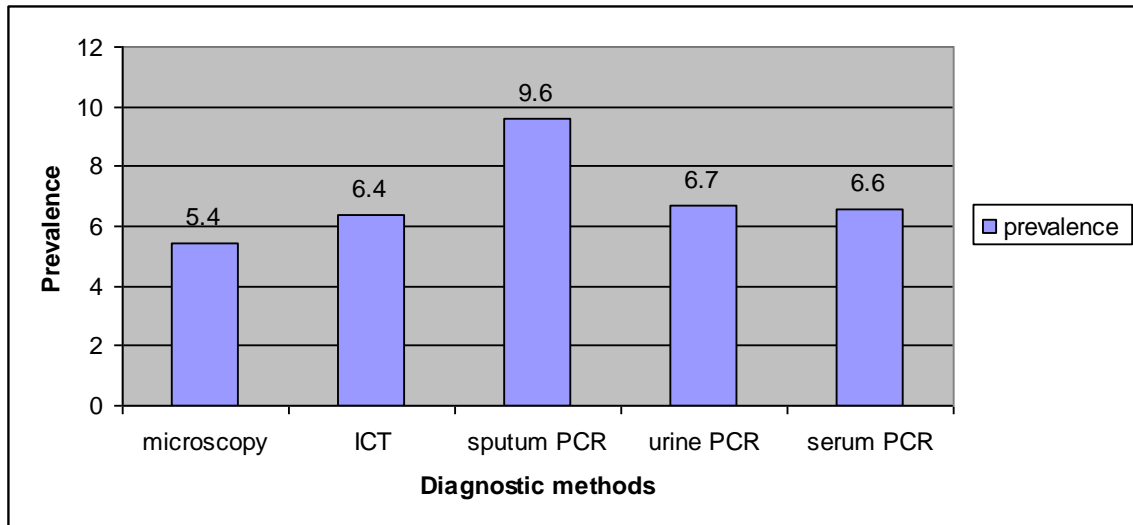
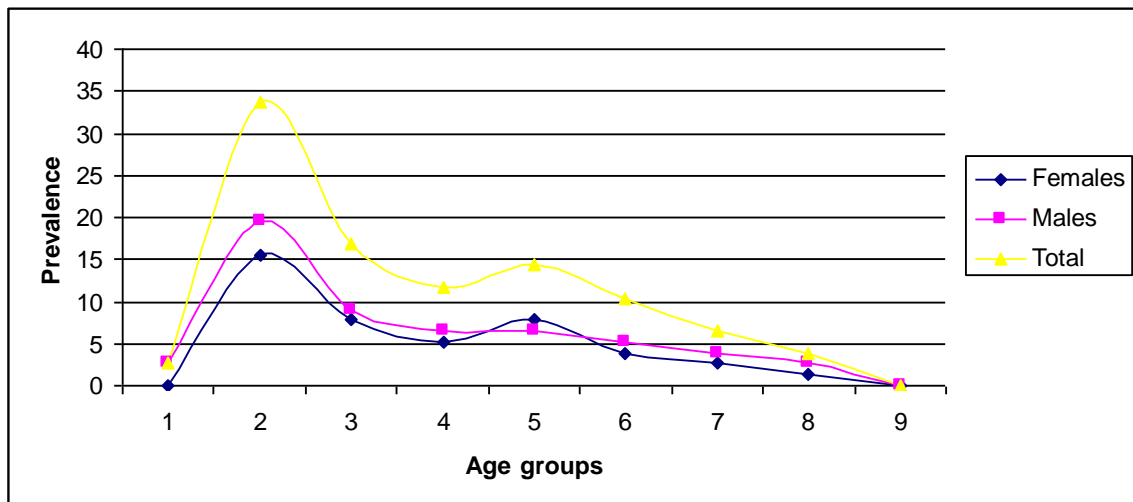


Figure 4.8: Prevalence of LF in the Tana Delta using the five diagnostic methods

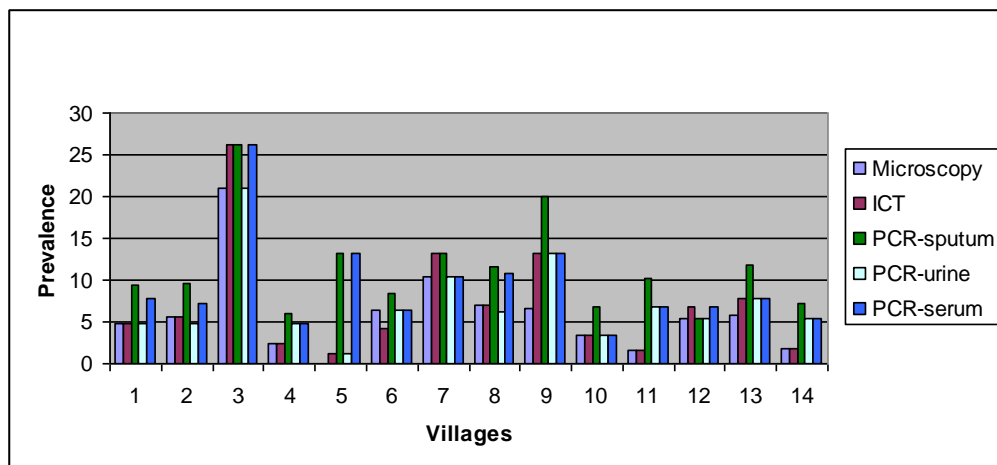


Age groups in years: 1=<10; 2=11-20; 3=21-30; 4=31-40; 5=41-50; 6=51-60; 7=61-70; 8=71-80; 9=>81

Figure 4.9: Prevalence of LF within various age groups using sputum PCR assays

The participants were divided into age groups in years as: group 1= <10; 2=11-20; 3=21-30; 4=31-40; 5=41-50; 6=51-60; 7=61-70; 8=71-80; 9=>81. As illustrated in figure 4.9, there were more *W. bancrofti* DNA positive cases in the age group 11-20 when analyzed 33.8% (26/77) compared to all other age groups (Figure 4.9). More males were positive than females, apart from age group 5 (41-50 years), where females had a prevalence of 7.8% compared to males at 6.5%, which was not statistically significant. Of the nine participants above the age of 81, none had *W. bancrofti* DNA.

Results from the villages revealed high prevalence (26.3%) in Chakamba in all the PCR assays performed, with PCR assay for urine showing similar results (21%) as those of microscopy. In nine out of fourteen villages, PCR assay for sputum demonstrated higher prevalence values than all other tests (Figure 4.10). For instant, in Furaha, the PCR assay for sputum had 20% (3/15) prevalence compared to PCR assay for urine and serum which had 13.3% (2/15) each. When compared with the traditional methods of microscopy and ICT (Figure 4.10), sputum assay results remained high in all the 14 villages.



1=Kilelengwani; 2=Ngao; 3= Chakamba; 4= Tarasaa; 5=Chamwanamuma; 6=Shirikisho; 7=Kipini; 8=Idsowe; 9=Furaha; 10=Maziwa; 11=Dalu; 12=Wem+a; 13=Matomba; 14=Odda

Figure 4.10: Prevalence of LF within the study villages using the five methods.

4. 4. Results from mosquito PCR assays

There were 3551 female mosquitoes obtained from the study area (Table 4.3). Kilelengwani village had the highest number of mosquitoes accounting for 48.8% of all mosquitoes studied, Ngao and Chakamba followed with 637 (17.9%) and 455 (12.3%) respectively, whereas Matomba village had the least, 0.2% (7/3551).

The mosquito species found in the study area (Figure 4.12) included *Anopheles* 40.3% (1434/3551), *Aedes* sp. 7.0% (249/3551), *Culex* sp 30.2% (1071/3551), *Mansonia* sp. 21.4% (759/3551) and *Falcabia* sp. 1.1% (39/3551). Frequency of the different mosquito species within the study villages (Figure 4.13) showed presence of all the four genera of mosquitoes apart from *Falcabia* species which were found only in Chakamba (3), Kilelengwani (8), Ngao (7) and Shirikisho (21). *Mansonia* species were also found in 9 villages out of 14. *Aedes* species were not found in Maziwa and Odda villages.

Table 4.3: Frequency of mosquitoes within the study area

Village	Mosquito Frequency	Percent
Chakamba	455	12.8
Chamwanamuma	215	6.0
Dalu	19	0.5
Furaha	47	1.3
Kilelengwani	1734	48.8
Idsowe	39	1.1
Kipini	63	1.8
Matomba	7	0.2
Maziwa	27	0.8
Ngao	637	17.9
Odda	20	0.6
Shirikisho	13	0.4
Tarasaa	256	7.2
Wema	19	0.5
TOTAL	3551	100

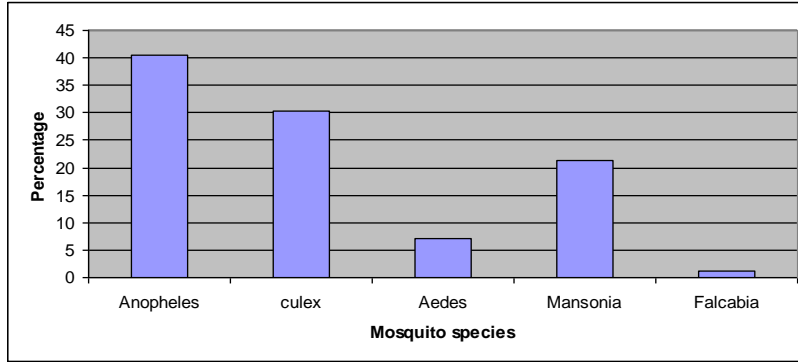
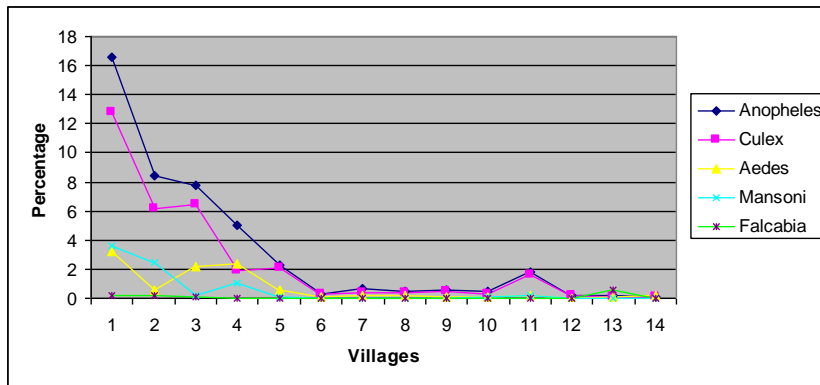


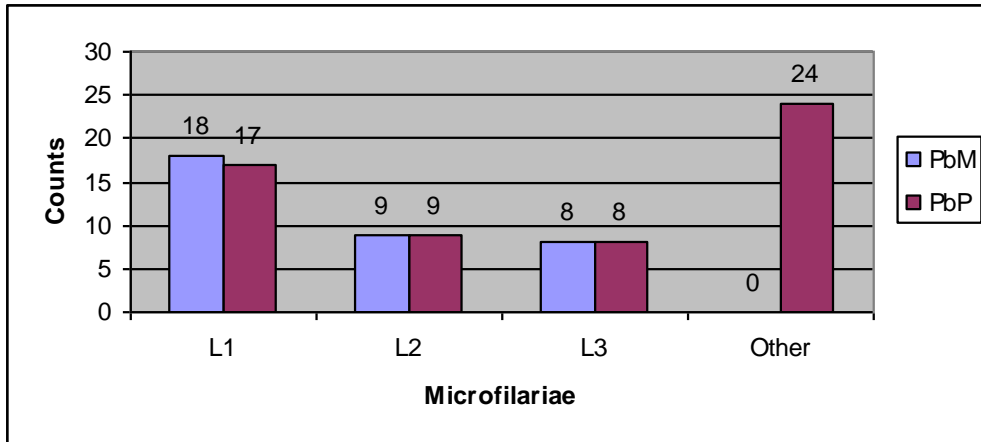
Figure 4.11: Mosquito species population in the Tana Delta



Key: 1=Kilelengwani; 2=Ngao; 3= Chakamba; 4= Tarasaa; 5=Chamwanamuma; 6=Shirikisho; 7=Kipini; 8=Idsowe; 9=Furaha; 10=Maziwa; 11=Dalu; 12=Wema; 13=Matomba; 14=Odda

Figure 4.12: Prevalence of Mosquito species by villages in the Tana Delta

Dissection and microscopy revealed microfilariae prevalence of 1.0% (35/3551) in all the mosquitoes dissected and a prevalence of 1.6% (58/3551) when the PCR mosquito assay was employed. Of the microfilariae observed (Figure 4.14), 0.5% (17/3551) were L1 larvae, 0.3% (9/3551) L2 and 0.2% L3. Twenty four mosquitoes (0.68%) which had not shown presence of microfilariae by microscopy, tested positive in the PCR assay (Figure 4.13). Six villages had *W. bancrofti* DNA positive mosquito when analyzed by PCR assay, compared to five villages which had MF positive mosquitoes by microscopy (Figure 4.13 and Table 4.4).



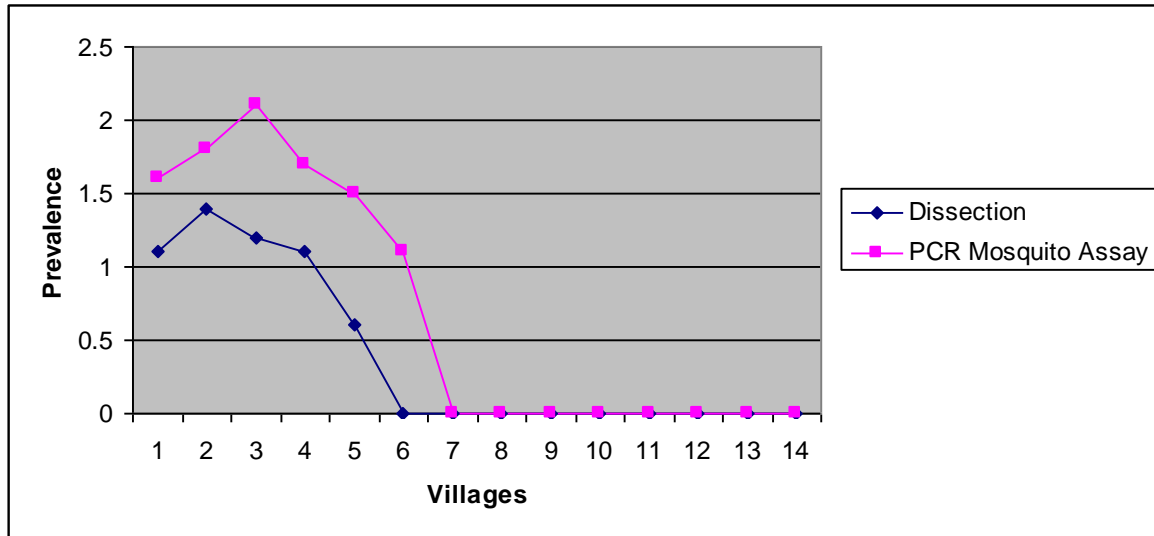
PbP=Positive by PCR; PbM=Positive by Microscopy

Figure 4.13: Prevalence of microfilariae by microscopy and PCR

A total of 12 mosquito species from the study area were identified. These included 4 species of *Anopheles* genera, 3 of *Culex*, 2 of *Aedes* and *Mansonia* and one of *Filcabia*. Mosquitoes dissected and observed by microscopy revealed 19 *Anophelesgambiesensulato(s.l.)*, (Table 4.5), 3 of *An. funestus*, 12 of *Culex quinquifaniatus*, and 1 *C.pipienshad* Microfilariae (MF). When PCR assay was performed, *W. bancrofti* DNA was detected in 27 mosquitoes belonging to *An. gambiae s.l.*, 7 of *An. funestus*, 14 *Cx. quinquifanciatus* and 7 of *Cx. pipiens*. There were two mosquitoes of *Culex* genera whose species could not be established that had *W. bancrofti* DNA.

When *W. bancrofti* infection in mosquitoes was analyzed by location, Kilelengwani had the highest number of infected mosquitoes at 14 (Table 4.4), constituting 40% (14/35) of all infected mosquitoes in this village, followed by Ngao (9), Chakamba (7) and Tarasaa (3), while Chamwanamuma and Shirikisho each had one

infected mosquito. While observing the larval stages as identified by microscopy (Table 4.4), the first stage larvae (L1), were 18 in total, with most found in Kilelengwani (7) and the least in Chamwanamuma (1). Larval stage 2 (L2) were 9 in total, while L3 were 8.



1=Kilelengwani; 2=Ngao; 3= Chakamba; 4= Tarasaa; 5=Chamwanamuma; 6=Shirikisho; 7=Kipini; 8=Idsowe; 9=Furaha; 10=Maziwa; 11=Dalu; 12=Wema; 13=Matomba; 14=Odda

Figure 4.14: Prevalence of MF in mosquitoes using microscopy and PCR mosquito assay within study villages

Table 4.4: Prevalence of Microfilariae infected mosquitoes within villages

Village	Total PbM	Total PbP	L1- PbM	L1- PbP	L2- PbM	L2- PbP	L3- PbM	L3- PbP	Other PbP
Kilelengwani	14	21	7	7	4	4	3	3	7
Ngao	9	11	4	4	2	2	3	3	2
Chakamba	7	12	3	2	2	2	2	2	6
Tarasaa	3	9	3	3	0	0	0	0	6
Chamwanamuma	1	2	0	0	1	1	0	0	1
Shirikisho	1	3	1	1	0	0	0	0	2
Kipini	0	0	0	0	0	0	0	0	0
Idsowe	0	0	0	0	0	0	0	0	0
Furaha	0	0	0	0	0	0	0	0	0
Maziwa	0	0	0	0	0	0	0	0	0
Dalu	0	0	0	0	0	0	0	0	0
Wema	0	0	0	0	0	0	0	0	0
Matomba	0	0	0	0	0	0	0	0	0
Odda	0	0	0	0	0	0	0	0	0
Total	35	58	18	17	9	9	8	8	24

PbM=Positive by microscopy
PbP=Positive by PCR assay

Table 4.5: Prevalence of *W.bancrofti* infection within mosquito species

Mosquito species	PbM	PbP		PbM	PbP
Anopheles			Aedes		
<i>gambie s.l.</i>	19	27	<i>Aegypti</i>	0	0
<i>Funestus</i>	3	7	<i>Furpurea</i>	0	0
<i>Chrityi</i>	0	1	Other	0	0
<i>Coustein</i>	0	0			
Other	0	0	Mansonia		
			<i>Africana</i>	0	0
Culex			<i>uniformis</i>	0	0
<i>Quinquefasciatus</i>	12	14	Other	0	0
<i>Pipiens</i>	1	7			
<i>Antennatus</i>	0	0	Falcobia		
Other	0	2	<i>uniformis</i>	0	0
			Other	0	0

PbM=Positive by Microscopy; PbP=Positive by PCR

While analyzing mosquito infection using the PCR assay, 21 mosquitoes in Kilelengwani had *W. bancrofti* DNA (Table 4.4) which was the highest number of infected mosquitoes compared to the other locations. Chakamba (12), Ngao (11), Tarasaa (9), Shirikisho (3) and Chamwanamuma (2) followed in that order (Figure 4.15). While examining larval samples recovered from the dissection, nearly all tested positive for *W. bancrofti* DNA, apart from one L1 larva, recovered from Chakamba, which tested negative. Other mosquitoes, which did not show larvae (24) when dissected, tested positive for *W. bancrofti* DNA (Table 4.4).

4.7 Analysis of population results

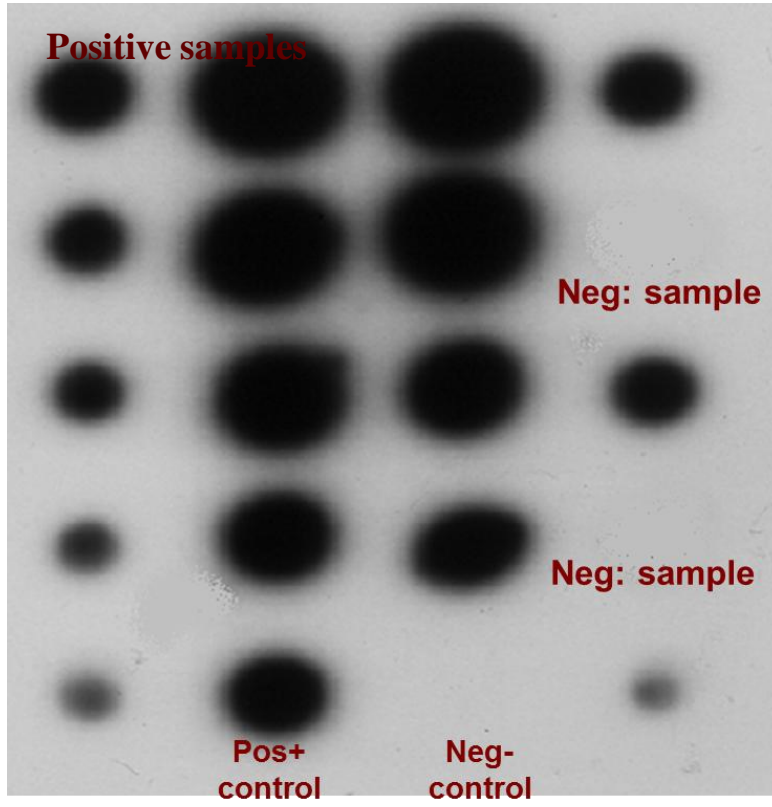
Statistical analysis was performed using SPSS V12. When lymphatic filariasis symptoms of edema, hydrocele and chest pains (Figure 4.7) were compared, the Pearson Chi-squared (χ^2) had a value of 310.070 and a likelihood ratio of 167.339 indicating no

significant differences ($p=0.05$). Similarly sputum versus serum and sputum versus urine χ^2 was 283.709 and 693.576, and likelihood ratio of 153.455 and 322.782 respectively.

Sensitivity of sputum PCR assay was calculated at 92.9 and specificity at 96.4. Positive predictive value (PPV) for sputum PCR assays was found to be 84.8%. The Negative predictive value (NPV) defined as the ability of a negative PCR assay to indicate *W. bancrofti* DNA was absent in the negative sample was calculated at 94.7%. Accuracy of the sputum PCR assay was defined as the proportion of all tests that gave the correct result as compared with ICT. The accuracy of PCR assay for sputum was 92.4%.

4.8 Quality control

Eighty human samples constituting 9.97% (80/802) of the study group were selected by picking randomly forty positive and forty negative PCR assay. Similarly, 360 mosquito samples representing 10.1% (360/3551) were selected randomly by picking 10 positive mosquito samples and 350 negative samples. All the selected samples from both Human and mosquitoes were subjected to ^{35}S dot blot hybridization. Positive results were determined by appearance of black dots on the photographic film (Figure 4.12). There was 100% concordance, indicating that in all these representative samples all the 50 (40+10) PCR positive samples had the specific *W. bancrofti* DNA, while the negative samples 390 (40 +350) did not have the specific *W. bancrofti* DNA.



CHAPTER FIVE: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

This study presents the first ever molecular epidemiology of *Wuchereria bancrofti* infections in the Tana Delta District of Kenya. Four modified PCR protocols employing body fluids and mosquitoes were used to study *W. bancrofti* infections in an identified population and mosquitoes of the Tana Delta. This is a novel development as the current diagnosis of lymphatic filariasis is still based on the traditional demonstration of microfilariae in peripheral blood collected at night by various parasitological examination techniques such as the counting chamber and other concentration procedures or serological card tests (Harinath, 1984; Wamae, 1994; Weil *et al*, 1998; WHO, 2002). This development presents sensitive and specific diagnostic assays which would be of great value in diagnosis of the nocturnally periodic *W. bancrofti* infection as traditional tests currently in use are not useful in diagnosis of cases with low microfilaraemia, occult or chronic infection (WHO, 2000).

Provision of such assays offers a fundamental requirement for effective lymphatic filariasis control (WHO, 2000; 2010) as elimination of lymphatic filariasis requires a precise and accurate diagnostic test for detection of *W. bancrofti* infection on mass scale in field surveys. The study offered assays which are able to provide sufficient knowledge of geographical foci of *W. bancrofti* infection, which is vital for efficiency in control and elimination of the disease. The assays provided accurate quantification of the status of the parasite system in the Tana Delta, which is paramount at any time-point to determine any change brought by an intervention program such as treatment (Figueroa, 1998). Reduction of parasite prevalence and parasite aemic loads in both patient and vector due to

mass treatment effects vector transmission and distribution of the infection cycle (Figueroa, 1998; WHO, 2000). This complicates the diagnosis efficiency of the available diagnostic techniques (Abassi *et al.*, 1999; Pani *et al.*, 2000) and further compounds the problems which exist in diagnosis of lymphatic filariasis (Wamae, 1998; Figueroa, 1998; Lucena *et al.*, 1996; Abassi *et al.*, 1999). This study provides four, essentially sensitive and specific mass diagnostic tests that can be used to collect *W. bancrofti* infection baseline data as well as surveillance of the disease during and after mass treatment.

5.1 Modification of the four polymerase chain reaction assays

The four modified Polymerase chain reaction (PCR) assays based on a highly repeated DNA sequence found in *Wuchereria bancrofti* (SspI repeat) used for mass diagnosis of bancroftian filariasis in the Tana Delta were found to be sensitive with the ability to detect the presence of as little as 0.04 pg of *W. bancrofti* DNA. The assays demonstrated specific amplification of parasite DNA from samples that were microscopy negative for the presence of the microfilariae of *W. bancrofti* suggesting that free circulating DNA was detected. The assays were also able to detect all the larval stages of *W. bancrofti*, including the third-stage infectious larvae (L3) from *Anopheles* and *Culex* mosquito vectors of the *W. bancrofti*, caught from the houses in the Tana Delta.

The assays sensitivity and specificity were in conformity with the findings of Abassi *et al.*, (1998) and Kagai *et al.*, (2008a and b), that demonstrated PCR assays were able to obtain higher prevalence of *W. bancrofti* infections by PCR sputum assay compared with microfilaria counts and ICT with the sensitivity and specificity of the

PCR sputum assay established as above ninety two percent and the predictive values and accuracy above eighty nine percent. In the current study, there was marked improvement in the DNA yield attributed to the superior DNA extraction methods of sputum and urine. By adjusting sample volumes and improving the incubation temperatures (Appendix 1) and introducing other changes such as the increment of ethanol precipitation time and a more enhanced evaporation system for the ethanol, improved the DNA. Urine samples are known to have less *W.bancrofti* DNA compared to serum (Lucena *et al.*, 1996) but a filtration technique adapted from Fischer *et al.*, (1998) enabled an enriched DNA yield. The PCR assays improvement may have also been achieved by use of Phusion DNA polymerase enzyme developed by Finnzymes® which is known to have a high fidelity. The enzyme, has 50 times more reliability than *Thermus aquaticus*Taq polymerase, and amplifies with more accuracy and speed, not attained with the standard PCR reagents. The processivity of Phusion DNA enzymes is approximately 10-fold greater than that of *Pyrococcus furiosus* (pfu) DNA polymerase and twice that of *Thermus aquaticus* (Taq) DNA polymerase. The dramatic increase in processivity results not only in shorter extension times, but more robust amplification and the ability to amplify long templates in a fraction of the time (Williams, *et al.*, 2007). Phusion DNA Polymerases also produces higher yields with lower enzyme amounts than traditional proofreading polymerases (Figures 4.2; 3; and 4). This improvement had important inference when the population and vector samples from the Tana Delta were tested against the traditional tests of microscopy and ICT and dissection of the mosquitoes.

5.2 Lymphatic filariasis in the Tana Delta

Providing new knowledge of presence of the *W. bancrofti* infections in the Tana Delta was necessary as the only records available indicate that the last surveys done in this area were in 1970s (Wijers, 1977). In this cross-sectional study, it was revealed that there was a higher gender ratio compared to the national figure of 1.01 (2011 Census estimates). The anomaly may have occurred due to the times when the samples were being collected (2200-0200hrs). It was noticed that the lactation of children by young mothers and insecurity issues could not allow some of the women to participate in this night exercise (Table 4.1). The study observed that the number of voluntary participant groups strongly corresponded to the size of the population. For instance, from Figure 4.2, Furaha and Chamwanamuma villages had the least participants while Golbanti had the most, which collaborated well with the households in the respective villages. Presumably, due to their high numbers in the Kenyan society, the age group 11-20 years had a high presence in the exercise. It is noted too that this young group seemed more adventurous and inquisitive of the study and this may have contributed to their presence in higher numbers compared to the other age-groups. The number of the aged people in the study area was relatively low. Life expectancy in Kenya stands at 54.9 years (World Bank, 2009) and it may explain why the least frequent age group was > 81 with a total of nine participants in all the villages.

5.3 Clinical, microscopy and ICT diagnosis of lymphatic filariasis

Clinical diagnosis, involving examination of the lymphatic filariasis symptoms was performed on all participants of the Tana Delta. In this study, three most common

symptoms for lymphatic filariasis, that is edema of the limbs, hydrocele and chest pains were observed in the study population. The edema of the limbs was found to be higher than the other symptoms ($p=0.05$) in the population. This was attributed to the presence of podoconiosis, a form of leg edema, prevalent in this area (Kelemu, *et al*, 2007). The Tana Delta is one of the few areas where podoconiosis and lymphatic filariasis exist in the same population. Whereas podoconiosis is associated with soils of the volcanic mountains of East Africa (Destas, *etal*, 2003), such as Ethiopian highlands, Tanzania's Kilimanjaro region and the Kenyan highlands located >2000M above sea level (ASL), in the study area, the disease appears in the lowlands 0-200M, ASL. The prevalent poverty in the study region makes the population to walk bare feet and being farmers work for long hours in their farms along the silted fertile river banks. The silt comprises of volcanic soils from Mt.Kenya region, which comes during flooding of the river in the rain seasons (Kibunja, 2002) providing the aluviate particles known to cause the condition.

Hydrocele caused by *W. bancrofti* infection is common in the coastal region, as described by Mwobobia *et al.*, (2000), who has worked on the south coast, and found that hydrocelectomies accounted for up to a third of the major operations recorded in the Coast Provincial General Hospital. In this study, the prevalence of hydrocele followed by edema, which compared well with concerns expressed in the Mwobobia (2000). No breast, penile or vaginal edemas were found during this study confirming observations by Mwobobia (2000).

Chest pain symptoms were included in the study because of Tropical Pulmonary Eosinophilia (TPE), a condition which is caused by *W. bancrofti* microfilarial presence in

the lungs. Probably because this condition is not as prevalent in Africa as it is in India, the chest pains as a symptom had the least frequency of the three symptoms observed.

Clinical diagnosis is challenging during the early stages of the infection due to absence of the physical symptoms, lack of characteristic fever and presence of many other tropical diseases with similar manifestations. The study deduced that total lymphatic filariasis prevalence using the clinical symptoms compared well with microfilariae examination, and perhaps where time or equipment is not available, clinical findings may be used when assessing approximate prevalence of the lymphatic filariasis.

Most of the traditional tests, microscopy and ICT, remain insensitive or logistically unattractive in cases of mass diagnosis. For instance, the traditional methods require microscopy of night blood, between 2200 and 0200 hours, which is inconvenient to both patients and the medical teams. Where medical teams have to perform mass diagnosis they risk insecurity, especially in the Tana Delta District where there are frequent inter-ethnic conflicts (Kibunjia, 2002). On three occasions during this study, the research team was forced either to obtain police escorts or limit the study in villages around the police stations. The card test, immunochromatographic, ICT is being used by many of the LF elimination programs. The card test can be used on blood during the day (Weil *et al*, 1996; Njenga *et al*, 2011) and has the convenience of short incubation period of fifteen minutes. The test however is not sensitive in cases of low parasitemia (Pani *et al*, 2000) and also in LF treated cases, where the test can remain positive for up to 36 months (Shuetz *et al*, 2000). These facts make these traditional tests unattractive in surveillance work, because the parasitemia as expected; reduces significantly after mass drug administration (Figuerola, 1998; Njenga *et al*, 2011).

Microscopy is the only diagnostic test available in the health facilities found in the Tana Delta. The findings of the microscopic diagnosis, in this study indicate a higher prevalence (5.4%) of lymphatic filariasis than that found in the neighboring Jilore town, situated 30 kilometers away. This shows an increase of 1.3% from a recent finding by Muturi *et al.*, (2008), who found 3.1% prevalence at Jilore town about 30 kilometers away, and the closest region where a recent LF prevalence has been done in recent years. The increased prevalence is probably occasioned by higher mosquito-human relationship. It should be noted that there is a close proximity of the studied villages to the water bodies which are breeding sites for the mosquitoes.

5.4 Molecular mass diagnosis of *W. bancrofti* infections in the Tana Delta

Earlier studies have found that microscopy, while being fairly specific, may not be sensitive (Abassi *et al.*, 1998 and Lucena *et al.*, 1996). This explains why the prevalence using all the four PCR assays was higher than that found while using microscopy. Higher prevalence found while using immunochromatographic test, ICT compared to microscopy may have resulted from detection of *W. bancrofti* antigens from either treated cases or from dead adult worms as reported by Shuetz, *et al.*, (2000) and Pani *et al.*, (2000).

Polymerase chain reaction, PCR sputum assays had a high prevalence, compared to both urine and serum. Although the results indicated differences in percentages, there were no significant differences statistically (Figure 4.8) between these results. The density of the sputum and high concentration of oxygenphilic microfilariae in the lungs (Hawking, 1967) could in part explain why the sputum assay had a high percentage. In

his paper, Hawking (1967), explained about the factor which holds up the passage of the microfilariae so that they accumulate within the lungs, in preference to the capillaries of other organs, as the great increase in oxygen tension. This may also explain even although the blood samples were collected between 2200 and 0200 hours, when the microfilariae appear in the circulation, the results suggest that there still could be a higher concentration of microfilaria in the lungs than in the blood circulation. As indicated by the prevalence found from the serum of the same patients. Another explanation why the serum had lower prevalence, may be the fact that when separating the serum from the blood, part of the microfilariae may have remained in the buffy coat after removal of serum. Similarly, the compromise of possible kidney filtration of whole or parts of microfilaria may have resulted to the low prevalence in urine assays.

Sputum appears like the ideal sample for diagnosis of lymphatic filariasis. Sputum however has a disadvantage because at the collection stage there is a risk of contamination with tuberculosis from TB patients whose prevalence may be as high as 30% of the population (WHO, 2010). Obtaining the sputum samples may also be challenging as emphasis is necessary in obtaining correct sputum sample and not saliva. Experience in the study was that the younger participants (<18years) had difficulties in producing sputum. By subjecting the participants to a jogging exercise, they were able to produce sputum. Older participants did not have trouble in sputum production. The inconveniences posed at the collection of sputum can be remedied, by use of protective gear for the medical teams, such as mouth pieces and gloves. Further, instructing patients

to control spread of droplets during collection by facing away from the person collecting the sputum and others.

5.5 Prevalence of *W. bancrofti* DNA and microfilariae in the study mosquitoes

Mosquitoes being the vectors of lymphatic filariasis remain an important aspect in the management of *W. bancrofti* infections (Goodman *et al.*, 2003). Although the numbers of mosquitoes caught in each collection method is not reflected in the methods, the number of mosquitoes caught in the CDC light trap was more, compared with the CDC gravid trap and the spray drop method. The CDC gravid method had the least number of the mosquitoes caught. The CDC light trap and spray drop method had many of other insects, such as beetles, flies and butterflies than the CDC gravid trap. There were approximately 20% of male mosquitoes caught in the CDC light and spray-drop technique, compared to the gravid trap which caught only female mosquitoes.

The mosquito species found in the study area included *Aedes*, *Anopheles*, *Culex*, *Mansonia* and *Falcobia* as identified morphologically using the taxonomic keys (Breeland and Loyless, 1989) this was in concordance with other East African studies that found *Anopheles* species as the most common mosquito (Simosen, *et al.*, 1999; Muturi, *et al.*, 2008, Onapa, *et al.*, 2007, and Beir, *et al.*, 1990). *Falcobia* species, known only to survive in the wild (Nelson, 1959), had the least prevalence with some of them caught indoors. Presence of these species indoors, demonstrates the nature of the poor housing in the area, some of the houses being only roofed shelters without adequate walls (Kibunjia, 2002) or structures such as doors and windows.

This study could not establish whether blood seen in the 95 fed mosquitoes was human blood or from other animals. Although the human-mosquito contact may have been high due to flooding and the generally poor housing, the percentage of fed mosquitoes appeared low according to an entomologist Ahmed Saidi, based in Tana River District (personal comment). This may have been due to the use of treated bed nets donated by various relief aid agents such as the Red Cross following flooding in the area.

Most of the filarial larvae found by microscopy were not necessarily those of *W. bancrofti* species. The mosquito PCR assays revealed that only 10 mosquitoes out of 63 which had larvae were positive for *W. bancrofti* DNA. According to Hashem and Badawy (2008) there exist other zoonotic filarial parasites, which can be transmitted by some species of mosquitoes (Orihel and Esslinger, 1973; Dissanaiké, 1975). One of these mosquito species, *Mansonia* has habits of biting both animals and humans (Onapa, *et al.*, 2007). *Mansonia* species constituted up to 17.5% of the mosquitoes examined in this study and may have contributed significantly to non *W. bancrofti* larvae (Figure: 4.13). The large number of mosquito species that can transmit the filarial parasites and the shared environment of humans and other animals probably account for the frequency with which humans are infected (Anderson, 1992; Simons *et al.*, 1974; Mendez and Bouza, 1988). Human infections with filarial worms found in other animals have been reported in many parts of the world and are found wherever the parasite is enzootic (Owen and Hennesey, 1932; Harbut and Orihel, 1995; Orihel and Eberhard, 1998). One of the zoonotic filarial infections is *Meningonema peruzzii* found in the African monkeys (Orihel and Esslinger, 1973; Pampiglione, *et al.*, 1995). It is likely that various species of

cercopithecoid monkeys including the redtail monkey common in Kenya are hosts (Orihel and Eberhard, 1998).

In this study there was no evidence of *W. bancrofti* DNA in the *Mansonia* species samples examined, confirming that the *Mansonia* species may not be a vector of *W. bancrofti* in the Tana Delta. This compares well with observations of Onapa, *et al.*, (2007), who examined the possible role of *Mansonia uniformis* mosquitoes in the transmission of lymphatic filariasis in an endemic area of Uganda. Although Onapa (2007) found both biting and feeding behavior were compatible with a potential for transmission, through further experimentation, his study concluded that *M. uniformis* had a limited potential to support development of *W. bancrofti* to the infective stage, and it does not appear to play a role as a vector under natural conditions.

5.6. Observations derived from the study

Although the results did not show statistical gender differences, the males seemed to show a higher prevalence than females, demonstrating that there could be an infection difference due to the type of dressing and occupation. Men in this region tend not to wear shirts and work outdoor more than women. This study demonstrated that the *W. bancrofti* infections exist in the Northern part of the Kenyan coast, re-establishing the fact more than 30 years after the last study was done by Wijers *et al.*, (1977). This was proved by all the tests performed, including PCR sputum assay which showed a high prevalence. Further, the vectors of LF exist in the region and positive mosquitoes were found through dissection and PCR assay. The furthest village of the study area was Hewani where three participants were found LF positive. Other towns and villages to the north, such as

Mpeketoni, Hindi, Lamu and the adjacent islands need to be examined for presence of *W. bancrofti* infections. Although this study did not examine the prevalence of LF by ethnic affiliations, it was observed that most of the positive participants belonged to the Bantu groups of Pokomo and Giriama tribes, compared with the semites, Wardia and Orma, who did not demonstrate any LF positivity.

Community co-operation is essential in disease elimination efforts, such as the one underway for lymphatic filariasis. This study observed that the local people preferred giving samples of sputum and urine, but were apprehensive of being bled. The participants can easily be shown how to collect mosquitoes, sputum and/or urine samples and transport them to a central laboratory thus enhancing the communal participation and solving most the logistical problems encountered by visiting medical teams such as access to areas that cannot otherwise be reached by vehicles.

The cost of performing PCR assays remains high due to various reagents, equipments and manpower training (Chansiri and Phantana, 2002). Several researchers have suggested pooling of samples (Chantaeu, *et al.*, 1994) in mass diagnosis and epidemiological studies. For instance, Abbasi *et al.*, (1998) was able to pool 14 negative Israeli sputum samples with one positive sample and was able to detect the *W. bancrofti* DNA. Although pooling of samples was not tried in this study, further studies in this area are suggested, especially using the modified assays able to detect low DNA concentration, as a way of reducing costs of performing PCR assays. Pooling of samples by house hold for instance, may reduce costs of PCR assays probably by up to 90% as the average house hold in coast has an average of 10 persons. Malhotra *et al.*, (2003) and Weerasooriya, *et al.*, (2003) have shown a higher prevalence of *W. bancrofti* infection

among the children whose parents are infected. This would mean increased chances of patients capture among the households with *W. bancrofti* infections in a pooled sample. The positive households could then benefit by treatment of all members as recommended by WHO (1998). Individual patient could be identified for further follow up and treatment, by analyzing household members separately. Pooling of mosquitoes has also been suggested (Ramzy, 2003).

A relationship between LF prevalence in the population and vector prevalence was observed. By taking advantage of this relationship, estimation of LF prevalence in the population could be determined from the vector prevalence. This could lighten the burden of population studies and would be a noble idea to use the vectors in determination of the spread of lymphatic filariasis in the population. In a simple calculation in this study area, a vector (mosquito) prevalence of 0.28% against a population prevalence of 9.6% using PCR sputum assay could be done by dividing $9.6/0.28$. A factor ψ (Hebrewshin) equal to 34.3 is obtained. This factor ψ would be directly proportional to the disease prevalence in the population. Therefore when a vector prevalence is 0.28%, there would be a prevalence of 0.28ψ or $(0.28 \times 34.3) = 9.6\%$.

The age group 11-20 years was found to respond well in the study, with the best turn out of all participants. This group had the highest lymphatic filariasis prevalence too. It is also observed that non-invasive samples of sputum and urine could be used in mass diagnosis due to convenience in sample collection. Single sample analysis of either sputum or urine can be used in the studies, as there was no significant difference when both were used.

Mass diagnosis using the modified PCR assays could be performed bi-annually instead of annual testing to reduce expenses but with special attention being focused on determination of the end point of mass drugs administration, MDA.

5.7 CONCLUSIONS

This study provides useful baseline data for the LF elimination exercise in the Tana Delta which has just began. The data will be compared with other prevalence data from this study area every time a mass drug administration (MDA) is done. Two MDAs will be performed every year for the next six years (WHO, 2000). And by using the modified PCR assays which are both sensitive and specific, an end point (a prevalence of 0.1%) for the MDA will be easier to assess.

- i) Results from the study shows only the Bantu ethnic groups such as Giriama and Pokomo had *W. bancrofti* infection. There should be a study to establish why there is no lymphatic filariasis in the Cushitic tribes of Wardia and Orma in this LF endemic area.
- ii) The extent and effect of the use of mosquito net in the elimination of LF in this study area should be studied.
- iii) When assayed by PCR, the prevalence of *Wuchereriabancrofti* infection in the study mosquitoes was 0.28%, against 1.8% presence of filarial larvae in mosquitoes. This shows that not all filarial larvae found in the mosquitoes were *W. bancrofti*. A study to identify which other filarial worms are found in the mosquitoes of the Tana Delta should be performed.

- iv) Following the starting of the MDA, there is a need to follow up on this project and look at the post MDA status of lymphatic filariasis in the Tana Delta.
- v) There is need to establish the vector status following each of the MDA

5.8 RECOMMENDATIONS

This study recommends the following:-

- i) The use of PCR assays for sputum and urine in determination of end-point for MDA in the study area through bi-annual mass diagnosis.
- ii) Where there is necessity to do spot check on LF, the age group 11-20 years can be used to evaluate the disease presence in the population
- iii) A diagnostic laboratory capable of performing PCR assays for the diagnosis of LF should be set up in the Tana Delta District.
- iv) A surveillance and monitoring of LF using PCR assays should be performed in the Tana Delta District after every round of MDA
- v) Use of the vector to assess the extent of *W. bancrofti* prevalence should be included in the surveillance and monitoring of the LF in the district
- vi) An assessment of the effect of mosquito nets on the spread of LF should be performed.

5.9 SUGGESTIONS FOR FURTHER WORK

Due to the findings of this study, that there is lymphatic filariasis in the Tana Delta, the following studies should be carried out.

- i) Molecular epidemiology of lymphatic filariasis in the islands in the north coast of Kenya, including Ndau
- ii) Establishment of the extent of *W. bancrofti* infections in the regions around the Somalia border.

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Appendix 1**PREPARATION OF REAGENTS AND METHODS****DNA EXTRACTION AND PCR ASSAYS****1. MOSQUITOES****Reagents preparation****Homogenization Buffer**

0.10M Sodium Chloride	0.59g
0.20M Sucrose	6.84g
0.01M EDTA	0.37g
0.03M Trizma Base	0.36g

Mix with 100ml of sterile water

pH 8.0

Lysis Buffer(pH 9.2)

0.25M EDTA	9.28g
2.5% (w/v) SDS	1.88g
0.5M Trizma Base	6.03g

Make up to 100ml with sterile water

Grinding Buffer

4 parts of homogenizing buffer: 1 part of lysis buffer

Procedure

Grind mosquito samples in 100 μ l grinding buffer.

Warm at 65°C for 30 minutes in a thermo mixer

Add 14 μ l of 8M Potassium acetate and vortex

Cool on ice for 30 minutes or at 4°C

Centrifuge at 4°C, 15,000rpm for 10 minutes

Transfer supernatant to new/clean labeled eppendorf tubes

Add 200 μ l of absolute ethanol, vortex for 1 minute

Incubate at -80°C in the freezer for 2hrs or longer-overnight

Centrifuge at 4°C, 15,000rpm for 20minutes

Pour off supernatant (pipette 200 μ l and discard)

Wash twice with 70% alcohol (Add 200 μ l for each wash then spin at 4°C, 15,000rpm for

10 minutes and discard the supernatant)

Rinse once in 100 μ l absolute alcohol

Spin/dry in a micro concentrator for 1hr

Suspend in 50 μ l TE buffer, vortex

Leave at 37°C for 30 minutes (thermo mixer)

Freeze them for use when needed.

2. URINE SAMPLES

Collect 50ml of urine sample in a sterile container. The sample should be collected in the morning and should be the midstream clear catch in 0.2M EDTA. Keep at 4°C.

Procedure

Filter about 20ml of urine through Nitran N45.

Transfer the filter in 0.5ml eppendorf vial on ice.

Add 500µl of 1M NaOH-triton (1%) solution.

Vortex the mixture and heat at 65°C for 30 minutes or leave overnight at room temperature

Read the pH using a non bleeding pH paper and adjust it to be 7-10 using 1M NaOH or HCl.

Centrifuge at 14,000rpm for 5 minutes at 4°C to remove the debris.

Transfer the supernatant to a labeled microfuge tube. Use screw cap tubes to avoid contamination.

Heat the mixture at 100°C for 5 minutes. Cool quickly on ice.

Add 800-1000µl of absolute ethanol.

Keep the samples at -80°C for 1-16 hours or overnight.

Centrifuge at 14,000rpm for 20 minutes.

Wash the precipitated DNA three times with 800-1000µl of 70% ethanol (pipette out 800µl and add 800µl of 70% alcohol for each wash).

Dry completely in a micro-concentrator.

Suspend in 50µl of TE buffer pH 8.0.

Store at -20°C for future use.

3. SPUTUM SAMPLES

Procedure

Put 200µl of sputum (mucoid) into a 1.5ml vial.

Add 200µl of 1M NaOH-triton (1%) solution.

Vortex the mixture and warm at 65°C for 30 minutes. Can also be left at room temperature overnight

Adjust the PH with HCl or 1M NaOH the pH to be at 7-10.

Spin quickly at 4°C, 14,000rpm for 5 minutes.

Cool quickly on ice.

Transfer the supernatant to new/clean tubes.

Add 800-1000µl of absolute ethanol.

Incubate at -80°C for 1-16 hours or overnight.

Spin at 14,000rpm for 20 minutes at 4°C centrifuge.

Wash thrice with 70% Ethanol, add (800-1000µl) for each wash and spin for 10 minutes at 14,000rpm and discard the supernatant.

Dry the DNA pellet in a micro concentrator for 1 hour.

Suspend DNA in 50µl TE buffer.

Heat in micro concentrator at 37°C for 30 minutes.

Keep at -20°C for future use.

NB: All procedures should be done on ice.

4. SERUM SAMPLES

Add 2µl of the serum sample into sterile eppendorf tubes.

Add 198µl of NaOH + 1% triton, and vortex.

Warm at 65°C for 30 minutes in a thermo mixer

Adjust pH to 8 with HCl 1:4 (dilution) or 1M NaOH.

Spin quickly at 4°C, 14000 rpm for 5 minutes.

Transfer the supernatant to new/clean eppendorf tubes.

Heat at 100°C for 5 minutes in a thermo mixer

Cool quickly on ice.

Add 400-500µl of absolute ethanol (or 2-2.5 times the volume).

Incubate at -70°C in the freezer for 1-16hrs or overnight.

Spin at 4°C, 14,000rpm for 20minutes.

Pour off the supernatant (pipette 800µl and discard).

Wash thrice with 70% alcohol (Add 800µl for each wash then spin at 4°C, 14,000rpm for 10 minutes) and discard the supernatant.

Spin/dry in a micro concentrator for 1hr.

Suspend in 50µl TE buffer, vortex.

Leave at 37°C for 30 minutes (thermo mixer).

Freeze them for use when needed.

POLYMERASE CHAIN REACTION (PCR)

Preparation of the template (DNA extracts)

Preparation of the master mix which should be enough for the samples you want to run.

Amplification of the DNA extract- (PCR thermocycler)

Detection and analysis of the reaction product through gel electrophoresis

Amplification of the DNA Extract

Mastermix preparation:

The reagents are put in a microfuge tube and mixed well;

10µl *Phusion* DNA polymerase buffer

0.5µl dNTPs

1µl primer 1 (direct primer)

1µl primer 11 (reverse primer)

87.5µl PCR water to top up to total volume of 100µl

NB: the final volume can be reduced to 50µ, 25µ depending on the kind of work being done.

Vortex the mixture

Pipette 90µl of the mixture to each of the labeled tubes

Add 10µl of the samples to each of the tubes.

Add 1µl of *Phusion* polymerase to each of the reaction tube and vortex.

Place the tubes in a thermocycler with the programme set and run PCR.

Add 5µl of ethidium bromide.

Set the gel tank with the combs on a flat leveled bench.

Pour the gel in the tank and let it set for 30-60 minutes.

Place the gel in an electrophoresis tank.

Remove the combs carefully and ensure that the 1X TAE buffer covers the gel completely.

Load 10ul of each amplification reaction onto the gel with 5µl gel loading buffer (containing bromo-phenol blue). Include a molecular marker 100 or 50 base pairs with target DNA.

Run the gel until the dye component has migrated into the gel for about 30-60 minute at 70-100 Voltage.

Visualize on U.V transilluminator and photograph the gel.

Check for appropriate band sizes in corresponded with the molecular maker as the predetermined DNA size.

Record the results in a record book or in computer programmes.

Interpretation of the Gel results;

a) Check the validity of positive or negative controls with respect to defined size in comparison with Molecular weight marker (188bp)

Negative control -Negative result – valid

Positive control -positive results – valid

Preparation of solutions for PCR and gel electrophoresis

TE buffer:

Mix one millilitre of 1M Tris pH 8 with 200 µl of 0.5M EDTA and complete the volume to 100ml with distilled water. Autoclave and store at room temperature.

TAE buffer: (50x)

Tris base Molecular weight 242g made to 1L

EDTA Molecular weight 372.24g make up to 1L

Preparation of a stock solution of 100ul of nucleotide mix

Mix 10µl of each nucleotide to 60µl of double distilled water

10µl ATP - Adenine

10µl CTP- cytosine

10µl GTP-Guanine

10µl TTP- Thiamine

60µl -distilled water

Mosquito identification and dissection

The collected mosquitoes are counted and identified into genera or species level based on the morphological characteristics under a standard dissecting microscope and with the help of taxonomic identification keys.

Place a drop of saline solution on a slide and a mosquito on the drop.

Using dissecting pins, the head, thorax and the abdomen are separated in search of larval stages of filarial worms which are identified as L₁, L₂ or L₃ by observation i. e L₁ is sausage shaped, L₂ is motile and short and L₃ is very motile, long and infective.

Dissection is done between 6-12 hours of the mosquito collection.

The filarial larvae are identified into species i. e *Wuchereria bancrofti*, *Brugia malayi* or *B. timori*.

Blood samples are collected after obtaining the National Ethical clearance.

The participants are selected according to selection criterion.

Written or oral consent is obtained from adult participants or from parents and guardians of children under age of 18years.

Blood collection is done by trained and qualified personnel.

The finger prick blood is collected in 100µl calibrated capillary tube for microscopy work and ICT test.

Venous blood samples are collected from the participants in sterile manner.

Swabs containing alcohol are used to sterilize the patient's skin before puncture.

The blood is collected in vacutainers containing EDTA anticoagulant.

100µl of whole blood is added into labelled vials containing 900µl of 3% acetic acid for microfilaria chamber count.

The venous blood is left to separate into serum/plasma and blood cells.

Serum is put in well labeled vials.

Mosquito collection

Mosquitoes are collected from participants' houses using, the gravid traps, CDC light traps or pyrethrum spray/ knockdown method after getting a written or oral consent. The mosquitoes are either dissected fresh or preserved under silica gel for molecular assays.

Pyrethrum spray catch or Knockdown method

White sheets are spread on the beds, chairs, under beds, and on the floor.

Pyrethrum sprays is applied in selected houses between 7.00pm and 10.00pm.

The knocked down mosquitoes are collected after 5-10 minute and put in labelled petri dishes in cool box.

Mosquitoes are transported to the laboratory for counting, sorting to species and dissection and then preserved under silica gel for PCR work.

CDC Light trap

- i) CDC light traps with a standard 6V 100 mA incandescent bulb and powered by 4 dry cell batteries are hanged from 6.00pm to 6.00am inside the rooms.
- ii) The trapping nets are removed from the traps carefully not to let the mosquitoes escape.
- iii) The mosquitoes are killed by exposing them to chloroform or insecticide.
- iv) The mosquitoes are sorted to species, dissected and then preserved under silica gel for analysis by PCR Assays.

Gravid Traps

- i) Gravid traps containing hay infusions to attract gravid mosquitoes for oviposition are used.
- ii) Grass infusion hay or rabbit pellets are used as mosquito oviposition attractant.
- iii) The infusion is prepared by composing the hay/pellets in water (0.5g hay to 114 litres of water) and incubated for 5 days.

The traps are placed in selected places/rooms in houses.

Mosquitoes are removed in the morning by first exposing them to chloroform and putting them in labeled petri dishes with moist filter papers in cool box.

The mosquitoes are sorted out in to species, a few dissected and the rest preserved in silica gel for analysis by PCR.

IMMUNOCHROMATOGRAPHIC TEST (ICT) FOR FILARIASIS

PROCEDURE

Remove the test card from the pouch just prior use

Label the card with the name or code number of the participant and the date of test.

Open the card and lay it flat on work surface

Add 100µl of whole blood onto the pink/white pad using a capillary tube from a finger prick or heel puncture. Serum/Plasma can also be used.

Wait for 30 seconds or one minute for the sample to flow into the pink area and wet the surface.

Remove and discard the adhesive liner.

Close the card. Press very firmly along the entire area to the right of the window, to ensure good test flow. Start timing.

Read the results through the view window after 10-15 minutes.

Positive sample

Wuchereria bancrofti antigen, complex with gold labeled PAb, and captured by MAb on membrane and forms pink line (T line).

The test is positive if two lines (T and C line) are seen in the viewing window. Any pink line in the T area indicates a positive test result. The test is positive even if the T line appears lighter or darker than the C line. This indicates the presence of *W. bancrofti* antigens.

Negative sample

NO gold labeled PAb is captured by MAb on membrane and no pink line forms.

The test is negative if only the C line is seen. To ensure that low positive samples have had sufficient time to develop, a negative result should not be recorded until 10 minutes have elapsed from when the card is closed.

Invalid Test Result

The test is invalid if the C line does not appear. If this occurs, the test should be repeated.

MICROSCOPY BY CHAMBER METHOD AND THICK SMEAR METHOD

Concentration method

This is concentration of low microfilarial in low level micro-filaraemia and for conducting filarial surveys.

Procedure

Dilute 1ml of blood in 9ml of distilled water and 1ml of commercial formalin.

Shake the suspension to hemolyze the red cells and centrifuge.

Discard the supernatant

Examine the deposit microscopically on a slide.

Counting Chamber

Clean the chamber gently.

Pipette 100ml of the serum preserved in 3% acetic acid.

Place it in the chamber (hollow part).

Count the present worms under a microscope.

APPENDIX 2**The Informed consent form****MOLECULAR EPIDEMIOLOGY OF *WUCHERERIA BANCROFTI* INFECTIONS IN THE TANA DELTA, KENYA****1. Participant information**

Name of patient _____

Date of birth: _____ Age: Sex: _____

Address: _____

2. Purpose of the study:

The overall aim of this study is to monitor in the Tana Delta, the infection of *Wuchereria bancrofti* (lymphatic filariasis) using sputum-PCR assay, urine PCR assay, microscopy and ICT. The study will also examine the prevalence of mosquitoes' *Wuchereria bancrofti* infection and compare infections in people and mosquitoes. This study is under the direction of Mr. Jim Kagai.

1. Procedure to be followed:

In this study, you will be clinically examined by a clinician and with the participation of local health workers. You may be asked to perform a short physical exercise after which you will give a sample of sputum. You will also be asked to donate urine and blood sample. These samples will be used for PCR tests indicated which will determine infection with *W. bancrofti*.

2. Risks

The risks involved are minimal. You will feel a bit of pain as you donate the blood sample.

3. Benefits

1) You will know from the results whether you are lymphatic filariasis positive or negative.

2) You will benefit from the treatment (DEC and/or Albendazole) for Lymphatic filariasis at the end of the study as recommended by WHO

3) Confidentiality of the records

Your medical records that are related to this study will be maintained in Confidentiality

4) PARTICIPANT'S* OR GUARDIAN'S* SIGNATURE.

*Tick the appropriate.

APPENDIX 3

Mosquito Traps



CDC Light trap for mosquito collection



CDC Gravid trap for mosquito collection

APPENDIX 4: Urine filtration equipment



IMVAC used in filtration of urine.