A COMPARISON OF TWO RECENTLY DEVELOPED MOLECULAR BASED METHODS FOR THE DETECTION OF *WUCHERERIA BANCROFTI* IN MOSQUITO VECTORS

By

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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 name.

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DEDICATION

This thesis is dedicated to my beloved husband, George Otieno Owuoche.

Baby, you rock my world!

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I thank the Almighty God for giving me life up to this moment to enable me carry out this research. In Him I live, I move and have my Being. A special thank you goes to all my supervisors for their invaluable support and input throughout the period of this study. I will forever be indebted to you. My sincere gratitude goes to the staff of Radioisotope Laboratory, Kenya Medical Research Institute, for their generous help with my project. You made it possible for me to successfully complete this project. I am grateful to my husband, sisters, brother and friends who went out of their way to encourage me and give me moral support as I conducted the research. Where would I be without your love? Words could never fully express the depth of gratitude that I feel for all your support.

May God richly and generously bless you all!

TABLE OF CONTENTS

CHAPTER TWO

ABBREVIATIONS AND ACRONYMS

ADL - Acute Adenolymphangitis

ATP - Adenosine triphosphate

BSA - Bovine Serum Albumin

^{o}C - Degrees Celsius

CDC - Centre for Disease Control

CFA - Circulating filarial antigen

DALYs - Disability adjusted life years

DDT - Dichlorodiphenyltrichloroethane

DEC - Diethylcarbamazine

DNA - Deoxyribonucleic Acid

dNTPs - Deoxynucleotides

EDTA - Ethylene diamine tetraacetic acid

g - Grams

GAPELF - Global alliance program for elimination of lymphatic filariasis

ICT - Immunochromatographic test

Ig - Immunoglobulin

IL - Interleukin

ITN - Insecticide treated bed nets

IVM - Integrated vector management

KEMRI - Kenya Medical Research Institute

LF - Lymphatic filariasis

MDA - Mass drug administration

mm - Millimeters

PCR - Polymerase chain reaction

pg - Picograms

pH - log H+ Concentration

PVP - Poplyvinyl pyrrolidine

QBC - Quantitative blood count

RNA - Ribonucleic Acid

RPM - Rotations per minute

SDS - Sodium Dodecyl Sulphate

SPSS - Statistical package for social sciences

SSC - Sodium Citrate-Sodium Chloride

SSP -Species specific primer

TAE - Tris acetate

TE Buffer -Tris-EDTA buffer

TPE - Tropical Pulmonary Eosinophilia

µl - Micro litre

UV - Ultraviolet

WHO - World Health Organization

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Different mosquito species incriminated as vectors for lymphatic filariasis include several mosquitoes in the genera *Anopheles, Culex* and *Aedes*. Monitoring the infection rates of these vectors may serve as an indicator of the disease in human populations. The classical method of diagnosis of infection in mosquitoes is dissection which is both insensitive and labour intensive. A cross-sectional study was carried out in three villages of the Tana Delta district to determine infection rates of *Wuchereria bancrofti* in mosquito vectors. Out of 100 female mosquitoes randomly selected from each of the three villages and dissected to determine larvae infection status, only 1 mosquito had third stage larva of *W. bancrofti*. In order to determine infection rates, two recently developed diagnostic tests: a PCR assay and a dot blot hybridization using radioactive probes were used to analyze DNA extracted from the individually dissected mosquitoes and to determine the proportion of mosquitoes positive for *W. bancrofti* infection. Results showed 2 out of the 300 mosquitoes analysed by PCR were positive for *W. bancrofti* infection. Analysis using ^{32}P dot blot hybridization gave results similar to those obtained by PCR based assay. There was no significant difference in the proportion of mosquito DNA samples positive for *W. bancrofti* infection using ^{32}P dot blot hybridization and using PCR based assay. Therefore, both PCR and ^{32}P dot blot hybridization are effective and efficient in detecting *W. bancrofti* infection in mosquitoes, making them useful as monitoring tools in the effort to eliminate lymphatic filariasis. The study also suggests that vector infection and infectivity rates could be used to determine the relative prevalence of the infection in humans from an affected area.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Background information

Filariasis is a disease affecting humans and animals and is caused by nematode parasites of the order Filariidae which are thread like worms (Ottesen and Ramachandran, 1995). Filarial parasites (Figure 1.1) are classified according to the habitat of the adult worms in vertebrate hosts. Cutaneous filariasis is caused by *Loa loa, Onchocerca volvulus* and *Mansonella streptocerca* while lymphatic filariasis by *Wuchereria bancrofti*, *Brugia malayi,* and *Brugia timori* (WHO, 1992) and body cavity filariasis is caused by *Mansonella perstans* and *Mansonella ozzardi* (WHO, 1992).

Lymphatic filariasis (LF) causes acute and chronic morbidity in tropical and subtropical parts of the world, affecting approximately 120 million people worldwide with approximately 1.2 billion people at risk of infection worldwide (Michael and Bundy, 1997). The global burden for LF is estimated at 5.78 million disability adjusted life years (DALYs) lost annually (Dreyer *et al.,* 1997). In Kenya filariasis due to *W. bancrofti* is endemic in the coastal districts with an estimated population of 2.5 million people being at risk of infection (Kasili *et al.*, 2009). After malaria that causes the greatest burden of disease as measured by DALYs, filariasis ranks second.

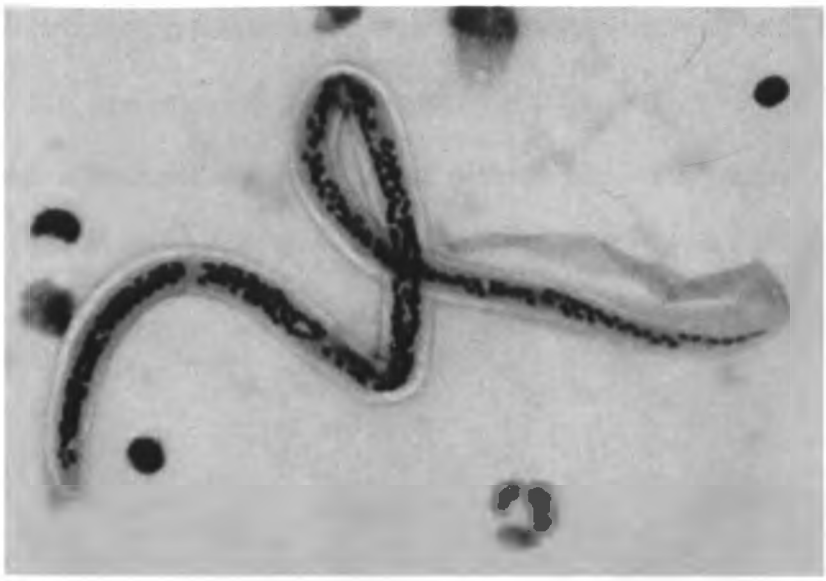

Figure 1.1: *Wuchereria bancrofti* microfilarial worm in a blood film.

Source: Lymphatic filariasis: the disease and its control, WHO Technical Report series. No 821, 1992.

1.2 Geographical distribution of lymphatic filariasis

Lymphatic filariasis is considered a disease of low-middle income countries and it falls among the neglected tropical parasitic diseases. It is associated with poor sanitation, industrialization and rapid urban population growth in the tropics. These conditions create suitable habitats for mosquito breeding (Evans *et al.*, 1993). Humans are the exclusive hosts for *W. bancrofti* even though certain strains of *B. malayi* can infect some felines and monkeys (McMohan and Simonsen, 1979). *W. bancrofti is* the most common cause of LF in Africa and accounts for approximately 90% of all infections globally (Michael and Bundy, 1997). *W. bancrofti* is endemic in Africa, India, Southeast Asia, the Pacific Islands, the Caribbean and South America (Babu *et al.*, 2005).

In Africa, the infections predominate in rural areas where access to basic health care is mostly inadequate. In Kenya, the only known type of LF infecting people is caused by *W. bancrofti* which is endemic along the coastal districts of Kwale, Malindi, Lamu, Kilifi and Tana River (Wamae *et al.,* 2001). *B. malayi* is limited to Asia and *B. timori* is restricted to some islands of Indonesia. Figure 1.2 summarises the global distribution of LF.

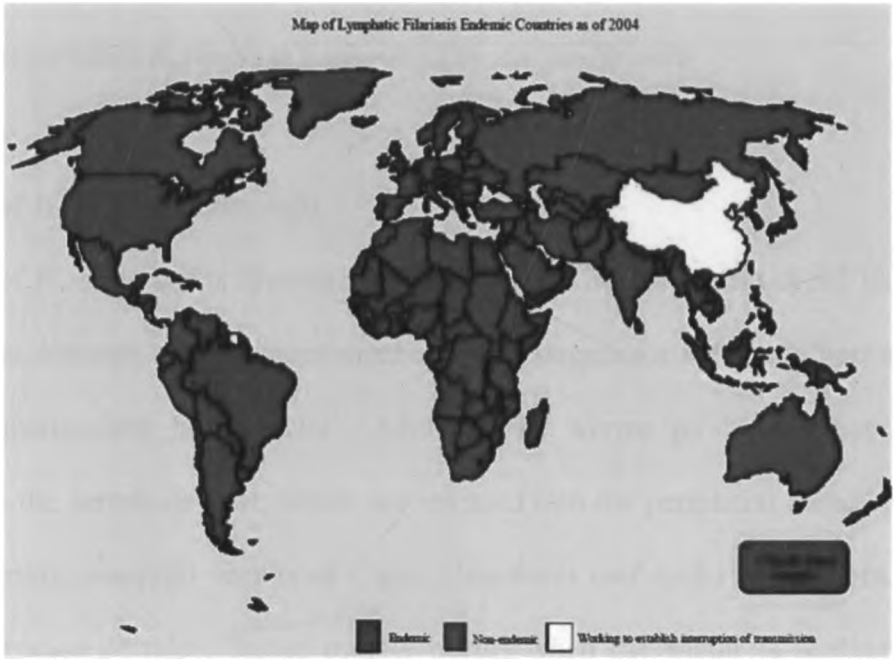

Figure 1.2: Map of lymphatic filariasis endemic countries as of 2006
Source: World Health Organization : Annual report on Lymphatic filariasis ; 2006.

1.3 Mosquitoes as vectors for lymphatic filariasis

Several species of *Culex, Anopheles*, *Aedes*, and *Mansonia* genera are involved in the transmission of LF (McMahon *et al.*, 1981). *Culex quinquefasciatus* is the major vector in Africa, Asia, and South America and transmits *W. bancrofti* nocturnally periodically.

C. quinquefasciatus has also been found to be the main vector of *W. bancrofti* in the coastal towns and the villages of Kenya (Wijers and Kinyanjui, 1977; Wijers and Kiilu, 1977). Culicine mosquitoes are important in most urban and semi-urban areas.

Among *Anophelines, An. gambiae* and *An. funestus* play a significant role in rural areas of Africa (Kasili *et al.*, 2009). Several species of *Aedes*, particularly *Ae. polynesiensis*, are the major vectors in the South Pacific islands where diurnally sub-periodic *W. bancrofti* is common. *B. malayi* is primarily transmitted by *Mansonia* and *Anopheles* species and some *Aedes* species while *B. timori* is transmitted by *An. barbirostris*.

1.4 Life-cycle of *Wuchereria bancrofti*

The life cycle of *W. bancrofti* is illustrated in Figure 1.3. The filarial life cycle, like that of all nematodes, consists of 5 developmental or larval stages in a vertebrate host and an arthropod or intermediate host (vector). Adult female worms produce thousands of microfilariae in the vertebrate host, which are released into the peripheral blood and are ingested by feeding mosquito vectors of *Culex, Anopheles and Aedes* mosquitoes. The highest concentration of microfilariae usually occurs when the vector is feeding most actively (Miranda *et al.*, 2005). A mosquito ingests the microfilariae during a blood meal. After ingestion, the microfilariae lose their sheaths to become first-stage larvae (L_1). Some of them work their way through the wall of the proventriculus and cardiac portion of the mosquito's midgut and reach the thoracic muscles. Here, they develop into second-stage larvae (L_2) and subsequently into third-stage (infective) larvae (L_3) in 7-21 days (WHO, 2002) depending on the ambient temperature and relative humidity.

The third-stage infective larvae migrate through the haemocoel to the mosquito's proboscis and can be inoculated into a human host when the mosquito takes a blood meal. During a blood meal, third-stage larvae (L_3) are inoculated back into the vertebrate via the skin through the bite of a mosquito, and then migrate to the lymphatics. Two moulting stages take place, first to L_4 and then to young adults which later, develop into mature adult worms in about 1 year and commonly reside in the lymphatics. When mature adults mate, the fertilized females produce microfilariae, which move from the lymphatics into the bloodstream. (McMahon and Simonsen, 1996).

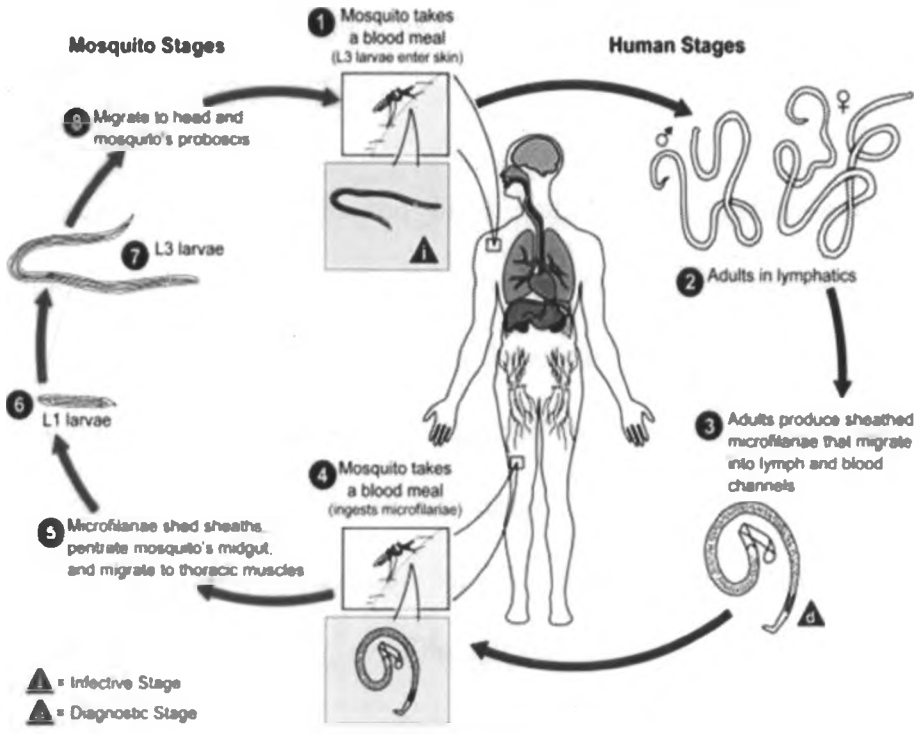

Figure 1.3: Life cycle of *Wuchereria bancrofti* in the host and the vector

Source: Elimination of lymphatic filariasis. An Interactive Guide for programme managers. The Wellcome Trust Publishing Group- International Health. 2004.

1.5 Pathology and clinical manifestation of lymphatic filariasis

Clinical features and pathology depend on the sites occupied by developing and mature worms, the number of worms present, length of infections and the immune responses of the host especially to damaged and dead worms. The pathology of LF is dependent on the pathogenic potential of the parasites, the tissue response of the host and external bacterial and fungal infections of which most of the pathology is limited to the lymphatics (Ottesen, 1980). The damage to the lymphatic vessels is mediated both by an immune response to the adult worms and by a direct action of the parasite or the products released by them (Vickery *et al.,* 1991). Clinical presentations are grouped into: asymptomatic (sub clinical), acute, chronic presentations and tropical pulmonary eosinophilia (TPE) of lymphatic filarial disease (Ottesen, 1980).

In endemic areas, a small proportion of the affected population does not show microfilaraemia or clinical manifestation even though they have some degree of exposure to infective larvae (Ottesen, 1992) similar to those who become infected. This is often referred to as the asymptomatic amicrofilaraemic stage of LF. In such circumstance, detecting the presence or absence of infections using diagnostic techniques is not possible. A considerable proportion of *W. bancrofti* infected people remain asymptomatic for months or years, even though they have large numbers of circulating microfilariae (Ottesen, 1992). Such people are important reservoirs of the infection.

Acute manifestations of LF occur in the form of acute lymphagitis filarial/Acute Adenolymphangitis (ADL), and these consist of intermediate episodes of lymphagitis, adenolymphangitis, funiculitis or epididymo-orchitis with fever.

The chronic form of LF includes elephantiasis, hydrocoele and chyluria (Ottesen, 1992). Elephantiasis affects the lower extremities causing disabling and disfiguring (chronic lymphoedema) of the limbs, breasts and the genital area. Hydrocoele condition which affects the male genitalia is fluid filled balloon–like enlargement of the sacs around the testicles which if left untreated can destroy the testicles. Hydrocoele is associated with *W. bancrofti* infections and is the most common clinical manifestation of LF (Estambale *et al.*, 1994; Wamae *et al.*, 1998). Chyluria, another form of the chronic filarial syndromes, is caused by the intermittent discharge of intestinal lymph (chyle) into the renal pelvis and subsequently into the urine (McMahon & Simonsen, 1996). 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 microfilarial stages of *W. bancrofti* (Ottesen, 1990). Microfilariae are absent in classical clinical manifestation but dead or dying microfilariae are demonstrated in lungs, liver and lymph node biopsies (Webb *et al.*, 1960). Patients present with paroxysmal cough and wheezing, low grade fever, scandy sputum with occasional haemoptysis, adenopathy and increased eosinophilia. It affects males twice as often as female and is rarely seen in children (WHO, 1992).

1.6 Diagnosis of lymphatic filariasis

Accurate diagnosis of LF still remains a problem. In general, diagnosis may be based on clinical, parasitological, histopathological or immunological approaches. Diagnosis of LF is done both in human populations and mosquito vectors (WHO, 1992; Ramzy *et al.*, 1997).

1.6.1 Microfilarial detection

Demonstration of microfilariae in the peripheral blood is the most common diagnostic method used in parasitological diagnosis of LF and is done on blood samples which are collected at night for nocturnal periodicity or during the day for diurnal periodicity. Several approaches have been used to demonstrate presence of microfilariae in blood samples including thick or thin blood smears, membrane filtration method, the counting chamber technique, Knott's method for concentrating microfilariae in low level microfilaraemia, DEC provocative test and Quantitative Blood Count (QBC) (Wamae *et al.*, 1994; WHO, 1992; McMahon and Simonsen, 1996). These methods differ in their sensitivity, cost and ease for use in field settings.

Effectiveness of detection of microfilariae in blood is hindered by the large number of infected individuals who are amicrofilaraemic (Chanteau *et al.,* 1994). The parasitological methods are affected by the nocturnal periodicity of *W. bancrofti* parasites which requires night blood collection (10.00pm- 4.00am) (McMahon and Simonsen, 1996; Miranda *et al.*, 2005) to coincide with microfilariae in peripheral blood.

1.6.2 Serologic assays

Detection of circulating filarial antigen (CFA) using immunochromatographic test (ICT) is regarded as the gold standard for diagnosing *W. bancrofti* infection (Weil *et al.*, 1996) and can detect filarial antigens in amicrofilaraemics, in clinical manifestations such as lymphoedema, elephantiasis and hydrocoele conditions (Weil *et al.*, 1997). According to studies carried out by Bhumiratana and others (1999), the ICT card test had a specificity of 100% when sera from microfilaremic subjects were positive, as when sera from *W. bancrofti* non-endemic subjects either with *B. malayi* microfilaremia or with other parasites, and those from normal controls were all negative by the test. When done in *W. bancrofti* microfilaremia sera, the ICT card test had a sensitivity of 100% using microscopy as reference.

The ICT method is easy to use in the field settings and acceptable by communities compared to night venous blood required by the other test methods (Njenga and Wamae, 2001). In addition, the antigen detection assays do not rely on the periodicity of the parasite. Test kit utilising Monoclonal antibody AD12 (Weil *et al.*, 1996) and Monoclonal antibody Og4C3 (Chanteau *et al.*, 1994; Lammie *et al.*, 1994; Weil *et al.*, 1996) are commercially available both detecting circulating adult worm antigens.

Although studies involving half life of circulating antigens in experimental rodents suggest a time scale of hours, it is uncertain whether these molecules can persist for longer periods of time in infected humans. Thus we cannot exclude the possibility that circulating antigens could be detected for days or perhaps weeks in individuals whose worms have been eliminated by chemotherapy.

Moreover, antigen detection is non specific due to cross- reaction with other helminth infections (Ottesen and Campbell, 1994). This limits the suitability of this method for use as a monitoring tool in the effort to eliminate LF. Figure 1.4 shows a photograph of an immuunochromatographic test card.

Figure 1.4: Immunochromatographic test card

1.6.3 Radiology based diagnosis

Ultrasonography using a 7.5 MHz or 10 MHz probe can locate and visualize the movements of living adult worms of *W. bancrofti.* The constant thrashing movements described as filarial dance sign can be visualized (Dreyer *et al.,* 1996). The structure and function of the peripheral lymphatic systems of the involved limbs can be assessed by lymphoscintigraphy after injecting radio-labelled albumin or dextran in the web space of the toes. The structural changes can be imaged using a Gamma camera (Ottesen and Campbell, 1994). These methods are not able to detect microfilariae thus are not useful in early detection.

1.6.4 Molecular based diagnosis

Recent advances in molecular biology techniques have been employed in designing species-specific primers for Polymerase chain reaction (PCR) based identification of parasites. For example, these techniques have been applied in the diagnosis of infections with the parasite genus *Plasmodium* that causes malaria in humans. Four species of the parasitic protozoan *Plasmodium* infect humans (*Plasmodium falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*); however, *P. falciparum* is responsible for most of the morbidity and mortality. Accurate diagnosis of *Plasmodium* species is essential for successful treatment. The small-subunit rRNA genes contain both genus- and species-specific sequences that were the basis of a probe assay and two different PCR-based assays to detect and discriminate between human malarial parasites. PCR has also been used to distinguish between different laboratory strains of *P. falciparum* and to detect mixed infections by analysis of polymorphic sequences encoding cell surface molecules containing regions of repeat sequences that vary in both length and sequence (Weiss, 1995).

1.6.4.1 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction is a rapid and specific method developed to detect parasite DNA in blood, mosquito and other specimen samples (Zhong *et al.*, 1996). It is a technique that amplifies a target DNA sequence. This reaction consists of a series of 20 to 40 repeated temperature changes called cycles, each cycle consisting of 2 to 3 discrete temperature steps. The initialization step during this reaction involves heating of the reaction mixture to a temperature of 94°C and reduces non-specific amplification during the initial stages of the PCR.

Denaturation step is the first regular cycling event and consists of heating the reaction to 94°C for 20 to 30 seconds, causing separation of the DNA strands by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA. During the annealing step, the reaction temperature is lowered to between 50°C to 65°Cfor 20 to 40 seconds, allowing complementary binding of the primers to the single-stranded DNA template. The temperature at the extension/elongation step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75°C to 80°C, and commonly a temperature of 72°C is used with this enzyme. In this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding deoxynucleotides (dNTPs) that are complementary to the template in 5' to 3' direction (Abbasi *et al.,* 1999).

Polymerase Chain Reaction techniques based on the identification of repetitive DNA sequences of species specific primer (*Ssp* I) (for *W. bancrofti*) have been developed.
The *Ssp* I repeat DNA family is dispersed, genus-specific, and exists in all of the different geographic isolates of *W. bancrofti* tested. The DNA family is conserved and has been shown to amplify *Ssp* I repeat DNA from different geographic isolates of *W. bancrofti* from around the world but not from other species of filariae or from human or mosquito DNA. With this PCR assay, the *Ssp* I repeat was detected in as little as 1 pg of *W. bancrofti* genomic DNA (about 1% of the DNA in one microfilaria) added to 100 µl of human blood (Zhong *et al.*, 1996). The specificity and sensitivity of the *Ssp* I PCR assay indicates that this approach has significant potential for improved screening of active *W. bancrofti* infection.

1.6.4.2 Blotting and hybridization

Blotting is a method of transferring proteins, DNA or RNA (ribonucleic acid), onto a nitrocellulose or nylon membrane and typically done following electrophoresis (Dyson, 1991). Following blotting, the transferred proteins, DNA or RNA may be visualized by different methods including colorant staining (for example, silver staining of proteins), autoradiographic visualization of radioactive labelled molecules (following hybridization) and specific labelling of some proteins or nucleic acids.

The most common blot applications used in modern laboratories are Northern blots, Southern blots, Western blots and Dot/Slot blots. Whereas Southern blotting involves transfer of DNA from gels onto the blotting membrane, Northern blotting transfer of RNA and Western blotting involves transfer of proteins.

Dot blotting involves application of sample DNA directly onto a nitrocellulose filter while bypassing gel electrophoresis process. The nitrocellulose filter is incubated with a solution containing a complementary probe, followed by washes to remove any unbound probe. Detection of hybridization is by autoradiography of a radio-labelled probe or by colorimetric reaction if an enzyme labelled probe is used (Feigin and Cherry, 1981).

The greatest success in the application of dot blot has been for diagnosis of DNA of viruses such as papillomavirus, cytomegalovirus, HSV (herpes simplex virus), parvovirus and hepatitis B virus (Evans and Kaslow, 1997). Dot blot has also been applied in studies of mutations and gene resistance with respect to malaria. However, dot blot assay has not been applied in studies to determine its effectiveness for use as a diagnostic assay in LF.

1.7 Management and control of lymphatic filariasis

Though LF is not fatal, the control of infections is very vital since chronic elephantiasis cases are not curable. Management of cases is important to avoid many working hours lost when one has episodic fevers and bacterial infections which worsen the situation.
For all patients, three issues should be considered. These are: anti-parasitic drug therapy, supportive clinical care and patient education and counselling.

To prevent possible transmission of LF to others, patients with clinical disease are treated with a regimen of anti-parasitic drugs. These include DEC, Mectizan® (generic name: ivermectin) and Albendazole (Palumbo, 2008).

Patients can easily learn hygiene measures that would be effective in minimizing infection and promoting lymph flow. These simple measures to be undertaken by the patient include washing the affected parts twice daily with soap and clean, cool water, and drying carefully, raising the affected limb at night, exercising the limb regularly and keeping the nails and spaces between the toes clean. Wearing comfortable shoes and using medicated creams or antibiotics to treat small wounds or abrasions is also helpful. Through these methods, even the worst case of elephantiasis can be improved over time (Palumbo, 2008).

Psychological counselling is also essential to support patients with LF-induced disability who can suffer from acute shame, isolation, sexual dysfunction and intense chronic pain and suffering.

1.7.1 Vector Control

With integration of the control of LF with other parasitic disease control programmes such as malaria, vector control has become more successful. Integrated Vector Management (IVM) uses a strategic approach of controlling mosquitoes in addition to the other LF control and management methods. Sustained vector control is achieved by use of several techniques which involves anti larval measures, anti adult measures and personal prophylaxis (WHO, 2004). The selection of control methods is based on the knowledge of the vector biology and disease transmission.

1.8 Justification

The World Health Assembly in May 1997 resolved to eliminate LF by the year 2020 through the Global alliance program for elimination of lymphatic filariasis (GAPELF) in endemic areas (Ottesen, 2000; Molyneux, 2003), using mass drug administration (MDA) with the combination therapy of albendazole and DEC or ivermectin (Chodakewitz, 1995; WHO, 2000). Monitoring of an intervention strategy is an essential component of the elimination programme which involves surveillance of either microfilaraemia or antigenaemia levels in the community or infection rates in the vector populations (Weil *at el.*, 1997). The process of xenodiagnosis is more acceptable to communities as it does not directly involve human populations like in the invasive procedures that involve obtaining specimens from humans to detect presence of an infection in an area. Furthermore, monitoring transmission in insects is ideal since mosquitoes offer a real time estimate of transmission rates of LF in affected population (Goodman *et al.,* 2003).

Traditionally, detection of *W. bancrofti* in mosquitoes requires dissection and microscopic examination of individual mosquitoes. Dissection is most suitable for monitoring mosquito infection levels when the infection level in the mosquito population is greater than 1%. When infection prevalence declines below 1%, the potential for missing mosquitoes infected with the earliest larval stages and for misidentifying the species of filarial larvae found within mosquitoes increases. Furthermore, as infection level declines, increasing numbers of mosquitoes must be dissected in order to demonstrate a significant decline in infection prevalence (Goodman *et al.,* 2003). Therefore more sensitive, rapid and specific tools are required.

Recently, two molecular based diagnostic tests have been developed for situations where microscopy may not be reliable. This study compares two molecular diagnostic techniques that are currently being studied in three villages of the Tana Delta district for detection of *W. bancrofti* infection. We further determine microfilariae rates in vectors that can be used as a direct measure of relative prevalence in human population from an affected area.

1.9 Objectives

1.9.1 General objective

This study was conducted to evaluate two recently developed molecular diagnostic tests for the detection of filarial infections in mosquito vectors, with the aim of determining relative sensitivity of these PCR based detection methods.

1.9.2 Specific objectives

1. To determine the proportion of mosquito DNA samples positive for *W. bancrofti* infection using polymerase chain reaction (PCR) based assays and ^{32}P dot blot hybridization.
2. To determine microfilariae rates in vectors which can be used as a direct measure of relative prevalence in human population from an affected area.

CHAPTER T WO

MATERIALS AND METHODS

2.1 Study area

The study was carried out at Tana Delta district of Coast Province, Kenya, which has an area of 16,013.4km^2 Tana Delta district is made up of Garsen, Kipini and Tarasaa divisions with the human population estimated at 134,000 (United Nations, 2007). Rainfall in this district is erratic, with rainy seasons in March-May and October-December. Flooding is regular, caused by heavy rainfall in upstream areas of the Tana River. The flooding leaves behind pools of water that provide suitable breeding grounds for mosquitoes. Figure 2.1 shows a map of divisions and villages in Tana River and Tana Delta Districts.

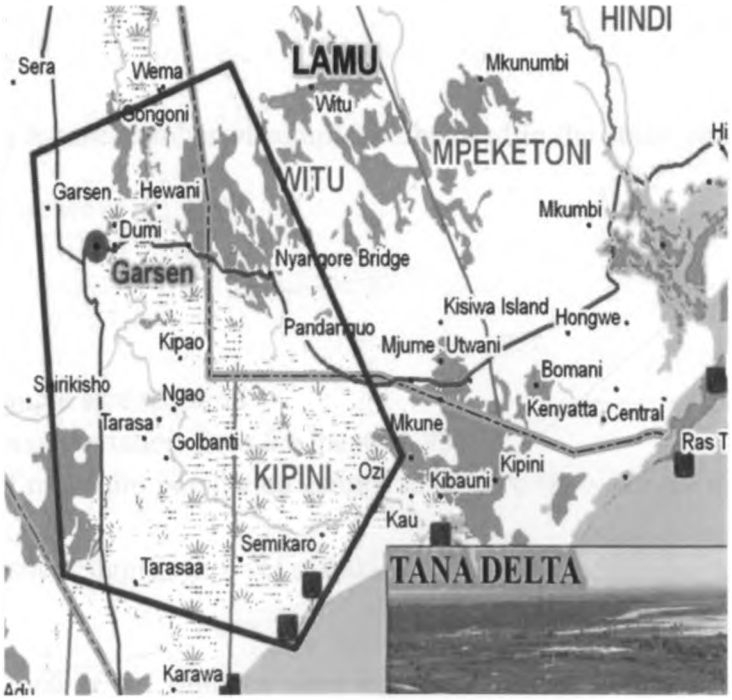

Figure 2.1: A map of Divisions and Villages in Tana River and Tana Delta Districts.

Source: Data Exchange Platform for the Horn of Africa. 2008

2.2 Study design

This study was curved out of a project by the Kenya Medical Research Institute (KEMRI) aimed at determining the prevalence of LF in Tana Delta district following MDA. Among the objectives of the study was to suggest suitable diagnostic techniques that could be used in the monitoring of this intervention strategy. To this end, a cross sectional study was conducted in three villages which were randomly selected. Six homesteads were randomly selected from each village by approaching each house after 3 kilometre interval. Mosquitoes were collected over 10 days during the month of May in the three villages of Tana Delta district, Idsowe, Chakamba, and Hewani. The villages were divided in such a way that the homesteads sprayed in the first week had traps set during the second week and vice versa.

2.3 Sample size

The sample size for the number of samples to be used in the study was determined using the formula of Fischer *et al.,* 1998;

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

Where,
n= minimum sample size required
z= standard normal deviation from the mean (1.96)
p= proportion of mosquito population estimated to have *W. bancrofti* infection
q= 1-p
d= level of statistical significance set at 0.05

Accordingly, a total of 101 samples were required. For this study 100 female mosquitoes were randomly selected from mosquitoes samples collected from each of the three villages.

2.4 Mosquito sampling

Mosquito samples were collected inside the houses of consenting household heads (see appendix 1). Three mosquito collection methods; CDC Gravid traps, CDC Light traps and Pyrethrum sprays were used in collection of mosquito vectors for analysis in all three villages.

2.4.1 Pyrethrum spray catch

Pyrethrum sprays were applied between 7.00pm and 10.00pm. White sheets were spread on the beds, chairs, under beds, and on the floor (1 room/house) and the rooms sprayed with pyrethroid insecticide formulation (Raid® Insecticide: Tetramethrin, Allethrin, Deltamethrin). The knocked down mosquitoes were collected after 5-10 minutes (WHO, 1975) and placed in labelled petri dishes in a cool box before being transported to the laboratory for further analysis. Further spraying of knocked down mosquitoes was performed to prevent them from escaping (as shown in figure 2.2a).

2.4.2 Centre for disease control (CDC) Light trap

A miniature CDC light trap with a standard 6V 100 mA incandescent bulb and powered by 4 dry cell batteries was hung from 6.00pm to 6.00am in each room(WHO, 1975). An example of CDC light trap that was used in this study is shown in figure 2.2c. The trapping nets were removed from the traps carefully not to let the mosquitoes escape and the mosquitoes killed by exposing them to chloroform. The mosquitoes were sorted, dissected and then preserved under silica gel for further analysis.

2.4.3 Centre for disease control (CDC) Gravid Traps

Gravid traps (shown in figure 2.2b) containing hay infusions to attract gravid mosquitoes for oviposition were used (Reiter, 1983). Hay infusion was used as mosquito oviposition attractant (Mboera *et al.,* 1999). The hay infusion was prepared by addition of 0.5g of hay to 114 litres of water and incubating for 5 days (Reiter, 1983).

Hay medium was changed every night to avoid bad odour generated from decomposition of hay reaching people in the rooms and which sometimes act as a repellent to the mosquitoes when used for several nights. Mosquitoes were collected in the morning and exposed to chloroform before being placed in labelled petri dishes with moist filter papers in cool box.

Figure 2.2a: Pyrethrum spraying of the knocked down mosquitoes.

Figure 2.2b: CDC gravid trap.

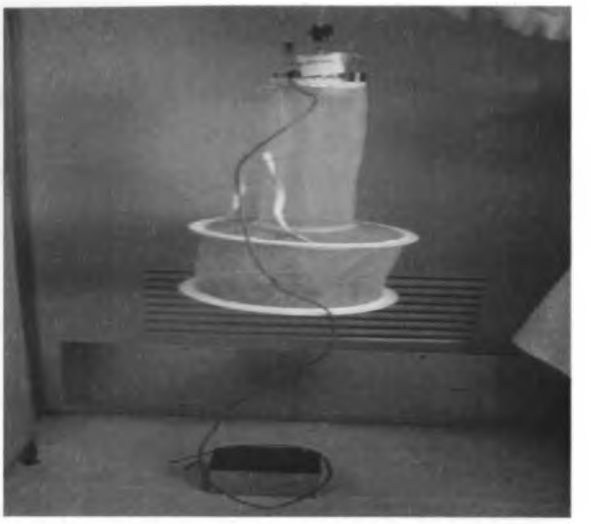

Figure 2.2c: CDC Light Trap.

2.5 Mosquito identification and dissection

All mosquitoes were counted and sorted. Female mosquitoes were identified under a standard dissecting microscope to genera level based on the morphological characteristics and with the help of taxonomic identification keys (Wharton, 1962; Peyton and Scanlon, 1966; Rattanarithikul, 1982; Gilles and Coetzee, 1987). Female mosquitoes were dissected under microscope to determine the presence and the larval stages (L_1, L_2 and L_3) of *W. bancrofti* in the mosquito. Dissection was done on saline solution and the larvae stages identified on observation. L_1 is sausage shaped, L_2 is motile and short and L_3 is very motile, long and infective (Chandler and Read, 1969). The larvae were stained using Giemsa to improve visualisation. Infectivity rate was given as a proportion of dissected mosquitoes with L_3 while infection rate was given as a proportion of dissected mosquitoes carrying L_1, L_2 and L_3. The larvae were preserved in silica gel for filarial species determination by PCR. Individual mosquitoes were packed and transported to Nairobi for further analysis.

2.6 Purification of genomic DNA from *W. bancrofti*

DNA was extracted using the ethanol precipitation method as described by Abbasi *et al.* (1999) with few modifications. Individual mosquitoes were homogenised in 100 µl of grinding buffer (see appendix 2) in labelled vials, with strict precaution taken to avoid contamination. The ground mosquitoes were heat at 65°C for 30 minutes in a thermo mixture and 14 µl of potassium acetate added and the mixture vortexed.

The mixture was cooled on ice for 30 minutes and the supernatant was transferred to new labelled vials to which a volume of 200µl of absolute ethanol was then added and vortexed. This mixture was left for 14 hours at -80°C. After 14 hours, the vials and contents were centrifuged at 14000 rpm for 20 minutes to pellet the DNA. The supernatant was carefully discarded and the DNA pellet was washed twice in 200 µl of 70% ethanol (For each wash, the mixture was centrifuged for 10 minutes at 14000 rpm). One final rinse was done using 100 µl of absolute ethanol after which the DNA pellet was dried in micro concentrator for 30 minutes. The pellet was suspended in 50µl of TE buffer pH 8.0 then vortexed and finally incubated at 37°C for 30 minutes before storage at-80°C for PCR. For quality control, clean *Anopheles* mosquitoes were obtained from the insectary at KEMRI. These were spiked with varying amounts of positive mosquito DNA product (1 µl, 5 µl, 10 µl and 20 µl). Extraction of DNA was carried out as described above, PCR product was obtained and gel electrophoresis carried out to determine the presence of bands.

2.7 PCR detection of *W. bancrofti* in mosquito samples

2.7.1 Reaction assay

The *Ssp*1 forward and reverse primer sequences designated NV1 and NV2 respectively and used in this study were 5′-CGTGATGGCATCAAAGTAGCG-3′ and 5′-CCCTCACTTACCATAAGACAAC-3′ originally designed by Zhong *et al,* (1996). The oligonucleotide primers are based on invariant non-coding DNA sequences of *W. bancrofti Ssp*1 repeat DNA sequence and generate a specific product of 188 bp with *W. bancrofti* DNA (Zhong *et al.,* 1996). Polymerase Chain Reaction was done in a total reaction volume of 60.5 µl containing 10µl template DNA, 0.5 µl of Taq DNA polymerase (KEMTAQ, Kenya), 5 µl of 10X PCR Buffer (LABSCO, Germany) to a final concentration of 1 X 25 mM $MgCl_2$, 1 µl 5pmol forward and reverse primers (LABSCO, Germany), 1 µl of 100 mM dNTPs mix (Promega, USA), and 42 µl of DNAse-free water (Sigma, USA). The amplifications were performed using Gene Amp® PCR system 9700 (Applied Biosystems Inc) and the cycling conditions were: Initialization at 95°C for 5 minutes, followed by 35 cycles of denaturation, annealing and extension at 95°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute respectively per cycle, plus an additional 10 minutes of final extension at 72°C after the last cycle.

2.7.2 Agarose gel electrophoresis of amplified DNA fragments

Horizontal submarine electrophoresis was used for analysis of amplified DNA. A 2% gel was cast by dissolving 3g. of molecular grade agarose (Sigma) in 150 ml of 1X TAE buffer. The mixture was heat at 90°C in a water bath until it dissolved completely and then cooled at 50°C in water bath.

The gel was poured in the gel tank and let to set for 60 minutes. The set gel was placed in an electrophoresis tank and combs were removed carefully to form wells to load the samples, with 1X TAE buffer being added to cover the gel completely.

A volume of 10 µl of PCR products were mixed with 2 µl of gel loading buffer and the mixtures were loaded into wells alongside a 50 bp DNA marker (Promega) and electrophoresed at constant voltage of 100V for 45 minutes. The gel was stained in 1µg/ml Ethidium Bromide and visualized on a 312 nm UV transilluminator and the gel image captured from Polaroid camera. Positive samples were identified as generating DNA amplicons of 188 bp. To confirm the results, the PCR assay was repeated for all samples.

2.8 Dot blotting and hybridization.

Dot blot and hybridization of the amplified samples was carried out using the method by Abdel-Muhsin and others (2002) with some modifications as described below.

2.8.1 Sample processing and blotting

Prior to blotting, the amplified DNA samples were denatured by adding 5 µl of 4.5M Sodium Hydroxide and 25 µl of 4M Ammonium Acetate to 20 µl of each DNA sample. Nitrocellulose paper was cut (13.5 cm X 8.5 cm X 0.45 µm) to fit the dot blot apparatus (BioRad, UK) and then soaked in double distilled water for 10 minutes followed by a second incubation for 10 minutes in 3M Ammonium Acetate before being placed onto the blotting apparatus. The blotting apparatus was then assembled and samples loaded into adjacent wells.

Transfer of the DNA onto the nitrocellulose membrane was achieved by a vacuum transfer apparatus connected to the dot blot apparatus. The membrane was air dried then baked in an oven for 2 hours at 60°C to covalently bind the DNA to the membrane.

2.8.2 Generation of radio-labelled probe

Radio-labelled probe was generated using Ready-To-Go T4 Polynucleotide Kinase. A volume of 25µl of sterile double distilled water was added to the tube containing the Ready-To-Go T4 Polynucleotide Kinase and the tube incubated at room temperature for 5 minutes. The contents were mixed by gently pipetting up and down. A volume of 11 µl of probe with a concentration of 25ng obtained from Hebrew University was added. A final volume of 12 µl of sterile double distilled water was added to bring the reaction mixture to 49 µl. Finally, a volume of 1 µl of [(γ-32 P]ATP(3000 Ci/mmol,10 µl) was added and then mixed gently. The tube was briefly centrifuged to collect the contents at the bottom of the tube and then incubated at 37 °C for 30 minutes in a thermomixer. The reaction was stopped by adding 5 µl of 250 mM EDTA

2.8.3 Hybridization and washing of the membrane

To reduce non specific binding of probe, the nitrocellulose membrane was placed in a rotor hybridization bottle and incubated with 10ml of pre-hybridization buffer at 60°C for 2 hours in a Stuart scientific ™ hybridization oven, with agitation (see appendix 2). The radio-labelled probe prepared as described in section 2.8.2 was then hybridized to the membrane bound DNA at 65 °C for 12 hours. Un-hybridized probe was removed by washing membrane to high stringency at 60 °C for 30 minutes in 2×SSC, 0.1% SDS. The washing was repeated using 1×SSC, 0.1% SDS.

After final wash, the membrane was drained well, air dried and exposed to a Fuji RX medical x-ray film in an autoradiographic cassette, with a fast tungstate-intensifying screen at -80 ºC for 12 hours and the film processed using an automatic x-ray film developer. Following autoradiograpy, samples were scored as either positive or negative depending on whether specific hybridization signals were observed on the x-ray film.

2.9 Ethical consideration

Sampling for mosquito vectors involved access and gaining entry into people's houses. Before the study implementation, villagers were informed about the aim of the study. Willing participants gave consent for setting of traps and spraying of the houses with pyrethrum spray(see appendix 1). Ethical clearance for this study was obtained from the Scientific Steering and Ethical review committees of the Kenya Medical Research Institute (KEMRI/TR/11/15VOL. LXII).

2.10 Data analysis

The results were subjected to non-parametric McNemar test. Data were analysed using the SPSS version 16.0 statistical software, to determine if there was any significant difference in the proportion of samples positive for *W. bancrofti* infection using dot blot hybridization compared to PCR based assays.

CHAPTER THREE

RESULTS

3.1: Number and species of female mosquitoes collected from each village

Table 3.1 shows the number of female mosquitoes selected from each village. Mosquitoes collected were identified as belonging to four genera, namely, *Culex, Aedes, Anopheles* and *Mansonia* species with the majority (149 out of 300 mosquitoes) of the mosquitoes belonging to the genera *Mansonia.* All the 3 villages recorded low numbers of *Aedes* mosquitoes compared to the other mosquito species collected.

	Idsowe	**Chakamba**	**Hewani**	**Total**
Anopheles	20	5	16	41
Aedes	1	16	6	23
Culex	21	42	24	87
Mansonia	58	37	54	149
TOTAL	**100**	**100**	**100**	**300**

Table 3.1: Mosquito species collected from three villages along the Tana Delta

3.2 Proportion of *W. bancrofti* infections in mosquitoes as indicated by application of the *Ssp*1 gene PCR assay

Following PCR and gel electrophoresis, samples which yielded a 188 bp DNA fragment were taken to represent a PCR positive sample with samples scored as either PCR positive or negative. A representative example of a stained agarose gel is shown in Figure 3.1. The proportion of mosquito samples collected from the 3 villages and positive for *W. bancrofti* infection by PCR is summarised in Table 3.2. In both Idsowe and Chakamba village, only 1 *Culex* mosquito was positive. This represents a proportion of 4.76% and 2.38% of *Culex* mosquitoes collected from Idsowe and Chakamba villages respectively. *W. bancrofti* infections were not detected by PCR in all the mosquito species collected from Hewani village. No positive samples were detected in *Aedes* and *Mansonia* mosquitoes.

	Anopheles	**Aedes**	**Culex**	**Mansonia**	**TOTAL**	**PROPORTION**
Idsowe	0	0	1	0	1	4.76%
Chakamba	0	0	1	0	1	2.38%
Hewani	0	0	0	0	0	0%
TOTAL	**0**	**0**	**1**	**0**	**2**	

Table 3.2: Mosquito samples positive for *W. bancrofti* infection by the *Ssp*1 PCR from 3 different villages in Tana Delta District

Figure 3.1: Agarose gel electrophoresis of some mosquito samples following PCR amplification.

Agarose gel electrophoresis of representative mosquito samples (Lanes 3-9) following PCR amplification generated using *Ssp*1 primers. The position for the specific *Ssp*1 gene PCR product (188 bp) is indicated by an arrow. Lane 1 and 2 are the positive and negative controls respectively, while M is 50bp DNA molecular size marker.

3.3 Dot blot hybridization

All the mosquito samples collected from the 3 villages and subjected to PCR were further analysed by Dot blot hybridization and the results are summarised in Table 3.3.

There were no differences in the detection of *W. bancrofti* within mosquitoes when PCR and dot blot were compared. In Hewani village, using dot blot and PCR, no positive mosquitoes were detected.

	Anopheles	Aedes	Culex	Mansonia	TOTAL	PROPORTION
Idsowe	0	0	1	0	1	4.76%
Chakamba	0	0	1	0	1	2.38%
Hewani	0	0	0	0	0	0%
TOTAL	**0**	**0**	**1**	**0**	**2**	

Table 3.3: Mosquito samples positive for *W. bancrofti* infection using dot blot hybridization

Figure 3.2 below illustrates representative results obtained from dot blot analysis of mosquito DNA samples from Chakamba village. Wells A1 and B1 represent the positive and negative controls respectively. The remaining wells (C1 – F 4) are the representative mosquito samples. Well E3 represents a positive mosquito sample.

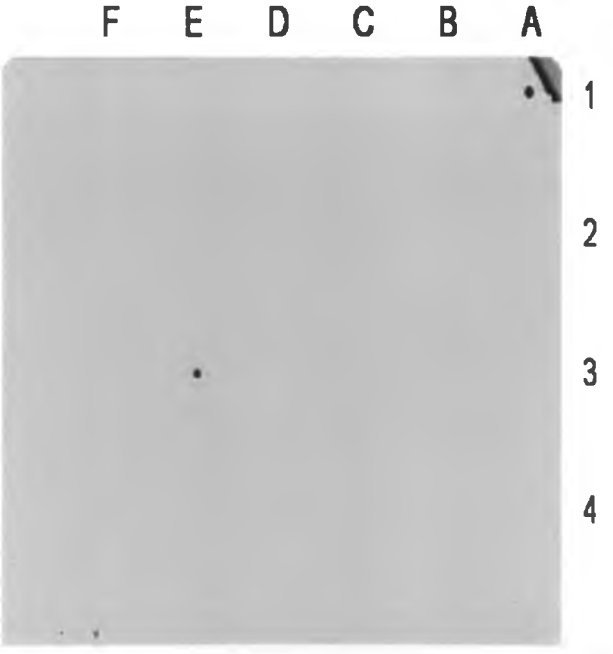

Figure 3.2: Dot blot analysis of mosquito samples from Chakamba village.

3.4 Larvae status of the dissected mosquitoes

Out of the 300 female mosquitoes dissected, only 1 *Culex* mosquito collected from Chakamba village was infected with third stage larva of *W. bancrofti* in its thorax giving an infectivity and infection rate of 0.33%. Table 3.4 is a summary of the number of mosquito samples positive for *W. bancrofti* infection in each village following dissection.

	Anopheles	**Aedes**	**Culex**	**Mansonia**	**TOTAL**	**PROPORTION**
Idsowe	0	0	0	0	0	0%
Chakamba	0	0	1	0	1	0.33%
Hewani	0	0	0	0	0	0%
TOTAL	**0**	**0**	**1**	**0**	**1**	

Table 3.4: Mosquito samples positive for *W. bancrofti* infection following dissection

CHAPTER FOUR

DISCUSSION, CONCLUSIONS AND FUTURE WORK

4.1 Discussion

Evaluation of transmission potential of the local mosquito population represents an approach that is minimally intrusive to residents of endemic areas. In addition, because of the relatively long period of time between exposure and establishment of infection (months) or development of lymphatic disease (years) in humans, monitoring of mosquito infection rates is likely to detect changes in transmission in a more timely manner (Bockarie *et al.*, 2000).

Monitoring the rate at which mosquitoes get infected with all stages of microfilariae through blood meal (infection rate) provides information that is useful in the evaluation of a control programme. However, it is also important to consider the proportion of mosquitoes infected with infective third stage larvae (infectivity rate) because not all the infected mosquitoes may produce third stage larvae and hence may not be potentially infective. Thus knowing the level of infectivity will give a real time estimate of transmission.

Morphological examination using microscopy has remained the mainstay for identification of infected mosquitoes. Although it requires minimal facilities and is inexpensive, microscopy lacks the sensitivity to detect low levels of infection. This is illustrated by the fact that in this study, dissection was able to detect only one infected *Culex* mosquito from Chakamba village.

In comparison, application of the *Ssp* 1 PCR assay and dot blot hybridization detected one more infected *Culex* mosquito from Idsowe village.

In endemic areas where mosquitoes may harbour more than one filarial species, microscopy is limited as it cannot identify different species. This is because all microfilariae belonging to different filarial species are morphologically similar, making it difficult to distinguish between species. After dissection was carried out in this study, the microfilaria obtained was subjected to a PCR assay analysis for species confirmation. Moreover, microscopy was found to be slow and labour intensive, especially since large amounts of samples were processed. It took me two weeks to carry out dissection on all 300 mosquito samples, carefully teasing the three body parts apart and searching for larvae in all those parts. PCR assay analysis of all the samples only took two days.

To this end, a more accurate assessment of infection in vectors would be valuable for quantification of epidemiological studies and in particular to allow estimation of transmission of parasites between hosts and mosquito vectors. The development of a sensitive and specific diagnostic assay for the detection of *W. bancrofti* infection in mosquito vectors would enable an accurate estimate on the proportions of infected vectors. This work reports the application of a PCR assay based on *W. bancrofti* species-specific sequence for the detection of infection in field collected mosquitoes. This assay was successfully applied to detect infections in mosquitoes, many of which may have infection rates far below the detection threshold by the conventional diagnostic method. The *Ssp* I PCR system used in this study is less labour intensive and less tedious compared to dissection.

The only labour intensive part of the process was homogenising mosquitoes in grinding buffer. The system is also species specific and was used to specifically detect the mosquitoes infected with *W. bancrofti.* Thus, the sensitivity and specificity of the *Ssp* I PCR system has demonstrated its potential use as a diagnostic tool.

A drawback to the PCR system used in this study is the use of electrophoresis and photo documentation equipment which are expensive. Moreover, this assay is species-specific and can only differentiate the filarial species but not various stages of the parasite in vectors.

Dot blot hybridization is a relatively novel molecular technique used in the detection of *W. bancrofti* infection in vectors. A complementary DNA probe was used for hybridization making it impossible to bind to other protein even if some protein was bound to the membrane. This greatly improved the specificity and sensitivity of the assay. The technique offered significant savings in time compared to other blotting techniques, as chromatography or gel electrophoresis steps were not required. Another advantage of dot blot hybridization is the ease of interpretation of results. Specific hybridization signals were observed on the x-ray film as a dot (positive) while the absence of a dot indicated negative results.

By applying the *Ssp* I PCR assay and comparing with dot blot hybridization using the non parametric Mcnemar test, the results obtained showed that there was no significant difference in the proportion of infected mosquitoes detected ($p > 0.05$), indicating that both techniques are sensitive in detection of filarial infections and thus will provide a more accurate assessment of infection compared to microscopy.

The extremely low levels of transmissions of LF in the vectors are likely to be linked to the use of different vector control measures by the community. For example it was observed that the use of insecticide treated bed nets (ITN), which was implemented by the malaria control programme, is widespread in the area. In addition, people practice traditional methods for controlling mosquitoes such as use of firewood smoke outside and inside the houses as a means of preventing mosquitoes from getting into the houses.

4.2 Conclusions and future work

The study showed that there is no significant difference in the proportion of samples positive for *W. bancrofti* infection using dot blot hybridization compared to PCR based assays. Furthermore, both PCR and dot blot hybridization were effective in detecting *W. bancrofti* infection in mosquitoes, making them useful as monitoring tools in the effort to eliminate LF. Since the majority of the mosquito samples collected belonged to the genera *Mansonia*, further studies should be carried out to determine whether these mosquitoes can be experimentally infected with *W. bancrofti* microfilaria and their role in transmission. There is also a need for research into development of an infective stage specific PCR assay to detect L_3 stage *W. bancrofti* in the vector. More research needs to be done on the development and performance of a rapid test strip for the detection of amplified *W. bancrofti* DNA using the *Ssp* I-PCR assay which does not require expensive laboratory equipment that might hamper efforts in filariasis endemic developing countries.

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APPENDICES

APPENDIX 1: INFORMED CONSENT FORM

EVALUATION OF TWO RECENTLY DEVELOPED MOLECULAR BASED FILARIAL VECTOR DIAGNOSTIC TESTS CURRENTLY BEING USED IN THREE VILLAGES ALONG THE TANA RIVER, KENYA

Participants Information

Name of the Household head: ----------

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

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

Investigators

Rose Atieno Kitoyi (Primary Investigator)

Dr. David Odongo (Co-Investigator)

Prof. Horace Ochanda (Co-Investigator)

Jim Kagai (Co-Investigator)

Institutional affiliations.

Kenya Medical Research Institute (KEMRI)

University of Nairobi

1. Introduction.

This is a research on lymphatic filariasis transmission in the vector mosquitoes. This study will be carried out by Rose Atieno Kitoyi who is a student at the University of Nairobi. There is no commercial interest in this study but the aim is to add knowledge on LF vectors and raise awareness to you as a community on the transmission of LF and vector control. The participation is voluntary and you have the right to refuse or to withdraw from the study without loss of benefits.

2. Purpose of the study.

The overall aim of this study is to collect mosquitoes inside your houses for the purpose of comparing PCR assays and those from P dot blot hybridization in detection of *W. bancrofti* infection in Tana Delta district, Coast-Kenya. This will involve indoor collection of mosquitoes using CDC light traps, gravid traps and pyrethrum spray methods. Thirty six house hold heads (participants) will be required. There will be a day for traps setting and another day for pyrethrum spraying for each of the selected house and this will take 10-12 days during the month of May.

3. Procedures

In this study you will be required to allow me and the field workers inside your houses especially the sleeping rooms for setting of traps and latter in another day allow us for spraying. You will also be asked not to interfere with the traps and during the day of spraying you will be requested to take foodstuff, water and young children outside or to another house.

4. Benefits

You and your community as a whole will benefit from free bednet which is one of the strategies used in vector control. Through National Programme for Elimination of Lymphatic Filariasis you will benefit from free annual Mass Drug Administration using DEC, Albendazole or Ivermectin drugs for LF treatment.

5. Risks

The risks involved here are minimal. Care will be taken during spraying to ensure that no sprays get into food stuff or in contact with young children by taking them outside or to another house prior spraying.

6. Confidentiality

Any personal details will be maintained in confidentiality. The mosquitoes will be coded in such a way that the infectivity rates of mosquitoes from each household of collection are not exposed.

7. Contact of Principal Investigator

This study is under the direction of ROSE ATIENO KITOYI ID no. 22477232 and in case of any problem, you (the participant) are requested to contact me at KEMRI, box 54840, Tel, 2722541 (Weekday, daytime) or on cell, 0723334768(all-time).

8. Contact of KEMRI/ National Ethical Review Committee

In case you need to enquire about your security and right to participate in this study, the involved institute will be KEMRI, box 54840, Tel, 2722541 (Weekday, daytime)

9. Compensation

There will be no losses or major risks involved in this study so you should expect no compensation.

10. Sample storage, exportation and further studies

Mosquitoes from the traps will be exposed to chloroform for killing. Mosquitoes both from traps and spray methods will be taken to the field laboratory setting for sorting, identification and dissection. The mosquitoes will be preserved under silica gel or 70% alcohol for transportation to KEMRI Radio Isotope laboratory-Nairobi where *W. bancrofti* DNA extraction will take place. DNA amplification (PCR) and PCR product analysis (Electrophoresis) will also be done. Dot blot hybridization will also be carried out on the samples.

11. Consent and signature options

Name of the Household head ----------

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

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

I---------- have fully capacity to consent. I have been informed about the study in details. Having read the information explained to me and understood it, I give consent to have traps be set in my house and spraying done in my house.

PARTICIPANT'S SIGNATURE OR LEFT THUMB PRINT

_____

Witness: I have witnessed that the above named participant whose thumb print is as above has given full consent to take part in this study.

Name……..

Signature…….....……..

APPENDIX 2: COMPOSITION OF REAGENTS AND SOLUTIONS

Grinding buffer

Grinding buffer is prepared by mixing homogenizing and lysis buffer in the ratio of 4:1 respectively. The homogenizing buffer was prepared by dissolving the following reagents in 100 ml of sterile double distilled water and adjusting the pH to 8.0: 0.10M Sodium Chloride 0.59g, 0.20M Sucrose 6.84g, 0.01 EDTA 0.37g, 0.03 Trizma Base 0.36g. Lysis buffer was prepared with the following reagents: 0.25 EDTA 9.28g, 2.5% (w/v) SDS 1.88g, 0.5 Trizma Base 6.03g; dissolved in 100 ml of sterile water and adjusting the pH to 9.2.

Pre-hybridization buffer

The hybridization buffer was made by mixing 6 ml of 20×SSC (Sodium Citrate-Sodium Chloride), 2 ml of 5× Denhardt's solution, 200 µl of Salmon sperm DNA, 200 µl of 10% SDS (Sodium Dodecyl Sulphate) and 11 ml of sterile double distilled water.

Denhardt's solution is made by mixing 2.5g Ficoll 400, 2.5g PVP (polyvinylpurrolidine), 2.5g BSA (Bovine Serum Albumin) and sterile double distilled water (upto 250 ml).