APPLICATION OF PCR TECHNIQUES UTILIZING SPUTUM AND URINE FOR MONITORING *WUCHERERIA BANCROFTI* INFECTION IN MALINDI DISTRICT, KENYA

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SEPTEMBER, 2004
DECLARATION

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

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I wish to dedicate this thesis to my wife Mrs. Teresia Mueni Mwaniki, my daughter Miss Ruth Wangari Mwaniki and my son Mr. Oren Kagai Mwaniki.

I express my gratitude to the following people...
I am greatly indebted to my supervisors Dr Eucharia U. Kenya and Dr. Faith Muli from the Department of Biochemistry and Biotechnology of Kenyatta University and Dr. Solomon Mpoke from the Centre for Biotechnology Research and Development, of Kenya Medical Research Institute (KEMRI), for their guidance and encouragement.

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<td>AIDS</td>
<td>Acquired Immune-Deficiency Syndrome</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CWRSU</td>
<td>Case Western Reserve State University</td>
</tr>
<tr>
<td>CBRD</td>
<td>Center for Biotechnology Research and Development</td>
</tr>
<tr>
<td>CFA</td>
<td>Circulating Filarial Antigen</td>
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<tr>
<td>DDW</td>
<td>Double Distilled Water</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diamine-Tetra Acetic acid</td>
</tr>
<tr>
<td>GPELF</td>
<td>Global Program on Elimination of Lymphatic Filariasis</td>
</tr>
<tr>
<td>HIV</td>
<td>Human Immune-deficiency Virus</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
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<td>IAEA</td>
<td>International Atomic Energy Agency</td>
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<td>ICT</td>
<td>Immunochromatographic test</td>
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<td>LF</td>
<td>Lymphatic Filariasis</td>
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<tr>
<td>MDA</td>
<td>Mass Drugs Administration</td>
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<td>NCP</td>
<td>Nitrocellulose Paper</td>
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<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PVP</td>
<td>Polyvinyl Purrolidine</td>
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<tr>
<td>SDDW</td>
<td>Sterile Double Distilled Water</td>
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<tr>
<td>SS DNA</td>
<td>Salmon Sperm DNA</td>
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<tr>
<td>SSC</td>
<td>Sodium Citrate-Sodium Chloride buffer</td>
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<tr>
<td>TAE</td>
<td>Tris- Acetate EDTA buffer</td>
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</table>
TE  Tris EDTA buffer
TPE  Tropical Pulmonary Eosinophilia
WHO  World Health Organization
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Lymphatic filariasis is caused by *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori*, and afflicts humans. The disease is prevalent in tropical countries, where 128 million are infected and 1.1 billion are at risk of being infected. Over 30% (38.4 million) of the people affected by lymphatic filariasis worldwide live in Africa. In Kenya, the disease is common in the coastal province where 2.5 million people live. The nocturnal *W. bancrofti* is the causative agent for lymphatic filariasis in Africa. These parasites are transmitted by mosquito vector, for which 77 species have been identified. The species belong to the genera, *Anopheles*, *Culex*, *Aedes*, and *Mansonia*. Specific and sensitive diagnosis of *W. bancrofti* infections has been one of the main challenges in filariasis research. To date, this objective has been hampered by absence of microfilariae in the later stages of the disease, inconveniences of nocturnal behaviour of the parasites, lack of a sensitive diagnostic method, and safer and easier sample collection procedures for mass diagnosis. In 1998 the World Health Organization, identified lymphatic filariasis as one of the diseases that can be eradicated. Several endemic countries including Kenya have put in place elimination programs. However, no surveillance methods have been identified for monitoring *W. bancrofti* infections during and after elimination programs. In this study, two PCR assays were applied to detect *W. bancrofti* infections in Mpirani location of Malindi district, coast province, Kenya. The traditional method utilizing examination of night blood for the presence of microfilaria under the microscope, and the immunochromatographic test (ICT), were also performed on the same samples. The positivity of *W. bancrofti* infection was found to be 22.0% (67/304) and 38.8% (119/304) respectively by microscopy and ICT, whereas sputum and urine PCRs positivity were, respectively, 42.8% (130/304) and 36.2% (110/304). The sensitivity and specificity of the PCR sputum assay was 97.5% and 92.4% respectively compared to 96.1% and 94.5% of PCR urine assay. Positive predictive values were 89.2% and 90.0% for PCR assay for sputum and urine respectively. Negative predictive values were 98.3% and 97.9% for sputum and urine respectively. Accuracy was 94.4% in PCR assay for sputum and 95.1% in case of urine. The study concludes that these PCR assays demonstrate great potential for consideration in both diagnosis and epidemiological studies of *W. bancrofti* infections. The study also recommends more studies in the use of urine in particular for surveillance of effectiveness of lymphatic filariasis programs.
CHAPTER ONE

INTRODUCTION

1.1 Background

Bancroftian filariasis is caused by a nematoid worm *Wuchereria bancrofti*. The life cycle of the parasite involves an arthropod and human. The human is considered to be the only definitive host for this parasite. The disease is widely distributed throughout the tropics, including Kenya where this study was carried out (Lindsay and Thomas, 2000). Bancroftian filariasis also referred to as lymphatic filariasis (LF), has a wide spectrum of clinical manifestations, most of which are asymptomatic (Beaver, 1970). Elephantiasis is a form of the symptomatic manifestation of the disease where, there is a great enlargement of the patient’s lower and/or upper limbs or genitalia. Genitalia enlargement forms hydrocoele in case of males and vaginal filariasis in females. Breasts may also be involved (Barry and Marquardt, 1996). Whereas the disease may not kill the victim, there is severe disfiguration and disablement, which cause enormous loses of social and economic production of the affected persons and communities (WHO, 1998).

Since the identification of bancroftian filariasis as a disease that needs to be eradicated by the World Health Organisation in 1993, a global program on elimination of lymphatic filariasis (GPELF) was set up in 1998, and most of the affected countries, Kenya included, have formed national lymphatic filariasis elimination programs. The major goals and strategies of GPELF include firstly, reduction of microfilaremia to interrupt transmission and prevent infection and secondly alleviation and reduction of the suffering of persons with filariasis.
Strategies to achieve the goals include mass treatment for four to six years of the entire at-risk populations with two drug regimens which will reduce microfilaraemia considerably, and thus reduce chances for mosquito transmission (Figuera, 1998).

Among the research needs of GPELF is development of surveillance tools, for epidemiological assessment and mapping as means for monitoring the effectiveness of interventions in reducing and interrupting transmission (WHO, 1998).

There are problems in diagnosis of bancroftian filariasis (Wamae, 1994), as the current tests are insensitive or logistically unattractive in cases of mass diagnosis. The traditional methods require microscopy of night blood, between 2200 and 0200 hours (Dennis et al., 1976, Denham et al., 1971, Knots, 1939). This is logistically challenging in cases of mass diagnosis, besides non-compliance by the communities involved, as they are sensitive to being bled (Lucena et al., 1998). The antigen test recommended by WHO uses day blood (Weil et al., 1996) but it has some shortcomings such as insensitivity in cases of low microfilarimae (Pani et al., 2000, Audrey et al., 2000) and may not be useful for surveillance in the post elimination phase due to insensitivity (Audrey et al., 2000). In this study, instead of blood, which poses risk of infection with blood borne diseases (Marcus, 1988), samples obtained through non-invasive procedures (Abbasi et al., 1999, Lucena et al., 1998) were used in PCR assays for detecting bancroftian filariasis.

1.2 Study Rationale

Lymphatic filariasis was identified as one of the diseases that can and need to be eradicated by the WHO in 1998. For effective elimination, the area identified need to
be effectively monitored for *W. bancrofti* infection, to establish baseline data for use in the elimination exercise. Further, the test to be used should be sensitive and specific for accuracy of such data. There is need to identify a better diagnostic test for surveillance of *W. bancrofti* infections during or after elimination (Audrey *et al.*, 2000). In a study carried out by Pani *et al.*, (2000), Njenga and Wamae, (2001) it was found that the WHO recommended antigen test (ICT) was unable to identify many of the positive cases of lymphatic filariasis. The patients also remain positive long after treatment of the patients (Audrey *et al.*, 2000). After mass treatment, the microfilaremia is expected to drop to very low levels (Addis *et al.*, 1997), thus the test identified should be very sensitive yet remain specific to avoid false positives.

In community studies it is vital to have good compliance of the community under study. One problem that has dogged laboratory-based studies is non-compliance especially where blood and other samples requiring invasive methods of collection are required (Mwobobia *et al.*, 2000). Polymerase chain reaction assays employing sputum and urine have recently been developed (Lucena *et al.*, 1998, Abbasi, *et al.*, 1999) but have not been applied, evaluated or validated in Africa.

This study sought to apply sputum and urine assays in monitoring *W. bancrofti* infections, as they combine the suitability of non-invasive sample collection methods with high sensitivity and specificity expected of molecular techniques.
1.3 OBJECTIVES

1.3.1 Main objective

The main objective of the study was to evaluate the utility of PCR based assays employing use of sputum and urine samples in the diagnosis of lymphatic filariasis, done by comparing with well established microscopy and ICT.

1.3.2 Specific objectives of the study

1. To establish *W. bancrofti* infection rate in the study samples by microscopy.
2. To establish *W. bancrofti* infection rate in the study samples by ICT.
3. To establish *W. bancrofti* infection rate in the study samples by PCR assays utilizing sputum and urine.
4. To compare the sensitivity and specificity of the PCR–assays utilizing sputum and urine.
CHAPTER TWO

LITERATURE REVIEW

2.1 History and distribution of lymphatic filariasis

Filaria has been known for thousands of years. The first documentation of this disease was found in Egyptian papyrus records dating back to more than 5000 years B.C. (El-Galley, 2003). The seventeenth century explorers in the Far-eastern countries of China, the Philippines, Borneo, Burma and Timor, recorded the presence of elephantiasis among the people living in those lands (Manson-Bahr and Apted, 1984). In 1900 Sir Ronald Ross of Liverpool School of Tropical Medicine reported that lymphatic filariasis is transmitted through mosquito bites (El-Galley, 2003). The filarial threadlike parasite *W. bancrofti*, which is one of the parasites that cause lymphatic filariasis, was first described in detail in the late nineteenth century (Manson-Bahr and Apted, 1984). It is the only member of the genus *Wuchereria*.

*Wuchereria bancrofti* is found both in mosquitoes and humans, which act as intermediate and the definitive hosts respectively. The sexual development of *W. bancrofti*, occurs in humans. Lymphatic filariasis is found in 80 countries, spread across the tropics and the sub-tropics (Figure 1), where most of the 128 million infected people live. Whereas 44 million infected people show deformation from the disease, over 76 million live with other forms of lymphatic filariasis without deformations. Kenya is among the 80 lymphatic filariasis endemic countries, with most infections found in the coast province, where approximately 2.5 million people live (WHO, 1998; Njenga and Wamae, 2001).
2.2 Life cycle of \textit{Wuchereria bancrofti}

The adult \textit{W. bancrofti} worms are located in lymph vessels near lymph nodes and in lymph nodes themselves. Adult females are viviparous and produce microfilaria (larvae), which reach the blood by penetration through tissues and blood vessels. The immediately released microfilaria, are referred to as pre-larvae (Manson-Bahr and Apted, 1984). The microfilaria can be observed in the blood circulation, between

Figure 1: The global distribution of lymphatic filariasis (adapted from WHO, 1998).
Infection
larvae in proboscis
L3 larvae
Development in mosquito
Ingestion

Adult worms
Microfilaria

Figure 2: The life cycle of *Wuchereria bancrofti* (adapted from WHO, 2000).
This is the infective stage for humans (Jeffrey et al., 1991). The slender L3 larvae migrate to the head of the mosquito and position themselves in the proboscis. Infection of the definitive host takes place when the mosquito takes another blood meal. The route of the infecting larvae is not completely understood, but they apparently migrate from sub-cutaneous tissue, to blood vessels and penetrate the lymph vessels where they mature and localize (Beaver et al., 1984). Adult worms are long and slender with a smooth cuticle and bluntly rounded ends. The head is slightly swollen and bears two circles of well-defined papillae. The mouth is small and the buccal cavity is lacking. The adult male is about 40 mm long and 100 μm wide. Its tail is finger-like. The adult female is 6 to 10 cm long and 300 μm wide.

The adult worms live intertwined together and after mating, produce numerous microfilariae which in-turn are taken by mosquitoes to perpetuate the life cycle (Faris et al., 1998).

Many thousands of these microfilariae are known to be shed, and being very motile, may penetrate through various tissues and organs (Beaver et al., 1984). The microfilariae have been found in liver, lungs, kidneys and urine.

2.3 Pathology of lymphatic filariasis

The pathology associated with W. bancrofti infection results from a complex interplay of the pathogenic potential of the parasite, the immune response of the host, and external ('complicating') bacterial and fungal infections. Several stages of the disease are recognized after the initial infection through a mosquito bite. The first stage is during the incubation period of lymphatic filariasis, which takes 3 to 12 months. There are no clinical symptoms seen in the patient. According to Wright and Baird
(1974), the microfilariae do not harm the human host and light infections may remain without symptoms though they are likely to be associated with eosinophilia.

The second stage occurs as an acute symptomatic period in which some lymphatic pathology (swelling of the extremities) may occur (Appendix 4) and this may be accompanied by pain, weakness of arms and legs, headache and insomnia. Fever is usually not present. Lymphatic pathology and dysfunction involve massive lymphatic dilatation around adult filarial worms, which remain fixed at characteristic sites in the lymphatic vessels (Ash, 1997). Histologically, dilatation and proliferation of lymphatic endothelium can be identified together with the abnormal lymphatic function associated with these changes.

Despite the paradigm that pathology associated with lymphatic filarial diseases is primarily the result of immune-mediated inflammatory responses, the above changes can occur in the absence of such overt inflammatory responses. These inflammatory responses can by themselves lead to both lymphoedema and hydrocoele formation. 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" (Ash, 1997).

Further to 'non-inflammatory pathway', there is filarial pathology that is mediated by host inflammatory responses. These responses can be initiated by immune reactivity or by bacterial and fungal super-infections of tissues with compromised lymphatic
function originating from filarial infection. Clinically these are seen as fevers associated with the early filarial infection.

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, but in the syndrome of tropical pulmonary eosinophilia (TPE) the pathogenesis is distinctly different. In TPE, there is enormous immunologic hyper-responsiveness where IgE and other pro-inflammatory molecules directed against microfilariae results in massive hyper-eosinophilia, allergic and other immunologic responses to those microfilaria-stage parasites. This causes them to be rapidly opsonized and cleared from the blood essentially immediately as they pass through the lungs. This causes severe pulmonary functional compromise and tissue destruction (Appendix 3) that leads to permanent lung disease (Ash, 1997).

The worms in the lymphatic system and patient’s immune responses cause tissue changes which restrict normal flow of lymph and result in swelling, fibrosis and eventually secondary infections in the affected tissues. Adult worms in the lymphatic vessels and glands lead to granulomatous changes and allergic tissue responses in some patients. The changes are associated with lymphangitis and temporary obstruction of lymphatic vessels for a few days. After many repeated attacks and secondary bacterial infections, permanent obstruction of a main lymphatic trunk may result. A progressive thickening and fibrosis of tissues follows after this. The lower extremities and groin are the parts most likely to be affected. The adult worms live for several years (Wright and Baird, 1974).
2.4 Diagnosis of lymphatic filariasis

Early clinical diagnosis is usually difficult especially in the tropics where fevers and lymphangitis are obscured by other parasitic diseases such as malaria. Clinical diagnosis is, therefore, supported by eosinophilia or intradermal test (Wright and Baird, 1974). Due to the duration it takes for the worms to get established in the lymphatics, demonstration of microfilariae can only be done 3-12 months post infection. Laboratory diagnosis of bacroftian filariasis is traditionally done by direct examination of night blood under the microscope for presence of microfilariae (Koneman et al., 1997). For specific identification of the microfilaria species, Giemsa
stained slides are necessary (Figure 4), so as to demonstrate characteristic position of posterior nuclei and the sheath (Ash, 1997). Although highly specific, the methods are logistically difficult to perform because of the requirement of obtaining blood samples at night when the microfilariae are in the blood circulation (Lucena et al., 1998, Zhong et al., 1996).

A variety of blood concentration methods such as the Knott’s technique (Knots, 1939) and membrane neocleopore -filtration procedures enhance detection of microfilaria (Denham et al., 1971, Dennis et al., 1976). Serological and immunological methods have been developed over time (Wamae, 1994). Most of these methods demonstrate either antibodies to *W. bancrofti*, antigens or excreted factors by the parasite in blood. There are many methods that detect antibodies to *W. bancrofti*, and these include complement fixation tests (CFT), immunohaemagglutination (IHA), and enzyme linked immunosorbent assay (ELISA).

Despite the use of blood, which is obtained by invasive methods, these tests have variable sensitivity and specificity (Moulia-Palat., 1992, Wamae, 1994, Chanteau et al., 1994), and may not be suitable for detection of early infection or in cases of low parasiteamia, observed soon after mass treatments (Manson-Bahr and Apted, 1984). These tests also do not distinguish between current and past infections (Weil et al., 1997).
Figure 4: Microfilaria showing the sheath and numerous nuclei.

Similarly, there are many tests that involve detection of antigens in the blood and body fluids such as hydrocele fluid and urine (Maizels et al., 1986, Lutsch et al., 1988; Weil et al., 1996). The detection of circulating antigens has the advantage of enabling daytime testing. One such test is the dipstick configuration test for rapid diagnosis of
lymphatic filariasis (Marsden, 1977). However, this test is not sensitive and does not discriminate between previous and current infections (WHO, 1998).

The Immunochromatographic test (ICT), which is a card test (Weil et al., 1997), is the 'gold test' for lymphatic filariasis, and detects circulating antigens (WHO, 1998). This test uses day blood but does not present maximal sensitivity for identifying microfilaremic patients (Weil et al., 1996). Pani et al (2000) found that ICT failed to detect 67% of individuals with microfilaremia counts of less than 10 per ml. Njenga and Wamae (2001), working on Kenyan patients, found that ICT kits could not detect 24.6% of patients. The test also has the disadvantage of using blood, for which trained manpower is required and the risk of blood borne infections (such as Hepatitis and HIV/AIDS to both patients and medical teams) is eminent (Marcus, 1988).

Detection of *W. bancrofti* DNA in patients is now possible due to the recently introduced PCR-assays, employing sputum (Abbasi et al., 1996), urine (Lucena, et al., 1998) and blood (Rawlings et al., 1994, Siridewa et al., 1996, Zhong, et al., 1996). The usefulness and adaptability of these DNA assays, for use in the endemic regions have not been extensively evaluated.

Ultrasonography can be used to demonstrate adult worms in the affected tissues (Amaral et al., 1994), and has been used as a method of diagnosis. However there are problems pertinent to the use of this method (Noroes et al., 1996a, Noroes et al., 1996b). To identify the adult worms by this technique requires a very high degree of
accuracy. Availability of this apparatus is limited in the economies of most of the countries affected by this disease and may not be suitable for large scale use.

2.5 Treatment of lymphatic filariasis

Treatment of lymphatic filariasis (LF) can be effected by surgery (Mwobobia et al., 2000) and/or chemotherapy (Addis et al., 1997). Diethylcarbamazine DEC (1-diethyl-carbamyl-4-methylpiperazine dihydrogen citrate) is the drug of choice. Given orally, DEC is absorbed in the gastrointestinal tract and reaches peak levels in blood after only 1-2 hours. The half-life in blood is 10-12 hours and it is excreted through the kidneys. Though the mode of action is not well understood (Simonsen and Dunyo, 1999), DEC acts rapidly by killing the circulating microfilariae, which later congest at the visceral blood vessels and get destroyed by phagocytic cells (Wright and Baird, 1974). Diethylcarbamazine (DEC) acts more slowly in adult worms. The recommended dosage by WHO is a total of 72mg/kg of DEC administered in a period of 12 days (Addis et al., 1997). It is well tolerated by the body and is effective in mass treatment where it is administered 1-2 times a year. It is microfilaricidal and studies have shown that 2 doses of 6mg/kg given in one week reduce microfilaremia by 97-99% in six months (Simonsen and Dunyo, 1999).

Diethylcarbamazine DEC has been used with success in Japan, Tahiti and to some extent in Kenya for mass treatment (Wijers, 1977). It can be incorporated in table salt as a preventive measure in endemic regions. When increased doses of DEC are administered, mild allergic reactions such as drowsiness, nausea and gastrointestinal upset, have been noted (Manson-Bahr and Apted, 1984). Other side effects are related to the killing of either microfilaria or adult worms. In cases of the killing of
adult worms, localized pain, inflammation, tender nodules, adenitis and lymphangitis have been recorded. A small percentage of patients may have lymphodema or transient hydrocele. These latter reactions are more associated with *B. malayi* infection as opposed to *W. bancrofti* infection.

Upon killing of microfilaria, systemic symptoms may be experienced by the patient. These include fever, headache, malaise, myalgia and haematuria. Adverse effects of DEC appear 48 hours after the first treatment and may last 1-3 days. The side effects are treated with steroids, as the anti-histamines are not effective (Addis et al., 1997). Although still rated as the drug of choice and very effective in individual treatment, compliance by the affected communities during elimination of lymphatic filariasis campaigns is usually compromised by the side effects of DEC treatment. Pre-education of the community is usually necessary before treatment with DEC is started.

Ivermectin is marketed as mectizan® by Merck and Company, USA. Ivermectin is a macrolide antibiotic and drug of choice in treatment of onchocerciasis. A single dose of 200-400μg/kg is known to have effect on the microfilaria. The drug, however, has no effect at all on the adult worms (Ismail et al., 1996). Ivermectin does not display adverse effects that are evident soon after killing of the adult worm by DEC. As such, Ivermectin is preferred for community lymphatic filariasis infection intervention programs. It has an advantage in the regions such as West Africa, where onchocerciasis is also found (Dreyer et al., 1995). Systemic side effects of Ivermectin are similar to those of DEC seen after killing of microfilaria.
Albendazole and benzimidazole have been found to have effects on the microfilaria. When co-administered with either Ivermectin or DEC, these drugs prolong suppression of microfilaria. The co-administration however also reduces efficacy of DEC on the adult worms (Dreyer et al., 1995). Some studies also have shown that albendazole may have effects on the adult worms when administered at 400μg/kg together with Ivermectin, and may improve the activity of Ivermectin (Ismail et al., 1996).

Suramin®, which is used in treatment of trypanosomiasis, has been tried and found to be effective in treatment of lymphatic filariasis (Manson-Bahr and Apted, 1984).

Radical surgery of the affected limbs or other parts of the body such as hydrocoele, followed by appropriate pressure bandaging and physiotherapy, reduces the swelling and in some cases the limbs have reverted to normal sizes. The results are however unsatisfactory in most cases, though plastic surgery looks promising (Addis et al., 1997). The high cost associated with plastic surgery may render this mode of after operative care not to be commonly applied.

Secondary infections occasioned by bacteria and fungi can be treated with antibiotics (such as amphotericin) and antifungals (Wright and Baird, 1974). Personal hygiene remains a prerogative for effective management.

2.6 Prevention and control of lymphatic filariasis

Preventive measures target either parasite or the vector. For prevention, DEC can be taken at treatment levels 1-2 times a year (Addis et al., 1997). Before DEC
administration, in the community, it is necessary to pre-educate the community on the side effects of the DEC, which are mainly related to body reactions after the killing of either microfilaria or the adult worm (Simonsen and Dunyo, 1999).


Ivermectin has reduced side effects, as it does not kill the adult worm. Ivermectin is the drug of choice in community programs due to high compliance from the communities and especially in places where onchocerciasis is prevalent such as West Africa (Gyapong et al., 2000). The global programme on elimination of lymphatic filariasis GPELF, focuses on treatment of lymphatic filariasis using combination of anti-filarial drugs, by mass drug administration (MDA) annually for five years (WHO, 1998). The GPELF hopes that this disease will be eliminated by the year 2020 (Figuera, 1998).

Health education and general hygiene is also considered beneficial in reduction of *W. bancrofti* infection. The mosquito vector, can be eliminated or highly reduced by use of appropriate insecticides, impregnated bed nets and eliminating the breeding sites (Bogh et al., 1998)
3.1 Study area and population

The study was carried out in Mpirani location situated approximately 25 kilometers from Malindi town (Appendix 1). The location has a population of 6,000 people (Government of Kenya census report, 1999). Most of the community is composed of the Giriama ethnic group, who occupy most of the coastal regions of Kenya. Most of the people are peasants, though a good number practice fishing and rearing of domestic animals. The area borders Athi River, which is known as Sabaki River locally. This is one of the two major rivers in Kenya. The Arabuko-sokoke forest and the Indian Ocean are within the vicinity and there is plenty of thick trees and bush vegetation. The presence of the river and the thick vegetation provide grounds for breeding of mosquitoes, the vectors for *W. bancrofti* (Bogh *et al.*, 1998). During the rainy season, most areas especially those near the river, such as Mbaoni, become flooded with water. The buildings in the study area are made of mud or sticks and thatched with coconut fronds or grass (Figure 5). Research activities were coordinated from the Marekebuni Health Center of the Ministry of Health. This site was chosen because KEMRI maintains an established field station there and the community is well acquainted with the KEMRI staff. Lymphatic filariasis is prevalent in this area (Wijers, 1977).
Figure 5: A typical house in the study area with high mosquito contact.

3.2 Inclusion criteria

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

3.3 Exclusion criteria

People who had not lived in the study area for the last three years were excluded from the study. Children below five years of age were also excluded due to logistical
problems of waking them at night when samples collection exercise was being carried out. People on active lymphatic filariasis treatment program were also excluded.

3.4 Samples

3.4.1 Samples collection

Each participant was examined by a clinician and asked to give blood, sputum and urine samples. The participants were also asked to sign a consent form (Appendix 6). Approximately 5ml of sputum was collected from each participant into a 15 or 50ml tube containing 5ml 0.2M Ethylene-diamine tetra acetic acid (EDTA). The EDTA was used as a DNA preservative. Where applicable, sputum was induced by deep cough following a brief jogging or push-up exercise (Abbasi et al., 1996).

Blood samples for microfilaria counts and ICT consisted of 1 ml of venous blood collected in 0.2M EDTA. The blood was bled between 22.00 and 02.00 hrs (Manson Bahr and Apted, 1984).

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 50ml tube, 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.
3.4.2 Sample preservation

The samples were collected in a final concentration of 0.1M Ethylene-diamine tetraacetic acid (EDTA) as a preservative of the \textit{W. bancrofti} DNA.

All samples were kept in cold boxes, before reaching the laboratory, where blood samples were kept at 4°C, and sputum and urine frozen.

3.4.3 Transportation and storage of samples

All samples were transported to the Malindi district laboratory within 2 to 4 hours in cold boxes. The sputum and urine samples were frozen to enhance preservation of the DNA, before transportation to KEMRI where the PCR assays were performed. Microscopy samples were transported in 3% acetic acid (McMahon \textit{et al.}, 1979) in cold boxes.

3.5 Sample size

The sample size was determined using the formula (Fisher \textit{et al.}, 1998).

\[ n = \frac{Z^2pq}{d^2} \]

Where;

\( n \) = minimum sample size required

\( Z \) = standard normal deviate from the mean

\( p \) = proportion of the study population estimated to have \textit{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,

\[
\frac{1.96^2 \times 0.2 \times 0.8}{0.05^2} = \frac{3.8416 \times 0.16}{0.0025}
\]

\[
= \frac{0.614656}{0.0025} = 245.8 \text{ or } 246
\]

Since the target population was less than 10,000 persons, then conversion for the population was calculated as follows:

\[
f_n = \frac{n}{1 + \left(\frac{n}{N}\right)}
\]

\[
= \frac{245.8}{1 + \left(\frac{245.8}{6000}\right)} = 244.4 \text{ or } 245
\]

Where, \(n_f\) = desired sample size when target is <10,000

\(n\) = Sample size when target population is 245.8

\(N\) = estimated size of population = 6,000

Thus, at least 245 persons were required for the study. The study recruited three hundred and four (304) persons and samples were collected from all of them.

### 3.6 Preparation of reagents

The reagents used for the study were prepared as shown in the appendix (Appendix 5). The chemicals and the reagents used in the preparation were of molecular biology grade, sourced from renowned companies such as Sigma inc., Invitrogen life sciences,
and GIBCO. The water (SDDW) was double distilled and sterilized. All reusable containers were cleaned in 0.35% calcium hypochloride, prolyteolytic soap and double distilled water (Ausubel et al., 1999). The equipments were rinsed with several changes of double distilled water.

3.7 Microfilaria counts

Microscopy was done as recommended by McMahon et al., (1979), where 100μl of patient’s whole night blood was added to 900μl of 3% Acetic acid, mixed and examined under low power in a microscope, using a chamber. All the microfilariae in the chamber were counted and recorded.

3.8 Immunochromatographic tests (ICT)

From each of the blood sample obtained, 100μl were 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. Presence of two bars indicated positivity while one bar indicated negativity (Weil, et al., 1997). The cards were retained at 4°C for any necessary references.

3.9 DNA Extraction

3.9.1 Sputum

The DNA extraction from sputum was carried out as described by Abbasi et al., (1999) 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 then vortexed and incubated at 65°C for 30 minutes. Subsequently, the
mixture was adjusted to pH 8.0, using 1M NaOH or 1M HCl. The mixture was then heated to 100°C for 5 minutes, cooled quickly on ice and spun briefly at 1500g for one minute to bring down all debris. The supernatant was transferred into a new microfuge tube. To precipitate DNA, 625µl or 2-3 times the volume of cold absolute ethanol added and the samples kept at -70°C overnight. The above samples were spun at 14000 rpm for 20 minutes in a microfuge centrifuge. The precipitated DNA was washed three times with 70% ethanol to get rid of extra precipitated salts. The tubes were then completely drained and dried in an Eppendorf Microconcentrator 5013™. The DNA was re-suspended in 50µl Tris-EDTA (TE) buffer, pH 8.0. Ten µl was used for amplification.

3.9.2 Urine

About 20ml urine was filtered through Nitran N45™ (Fischer et al., 2003). The filters, which are made of nitrocellulose paper, were then transferred into 0.5ml eppendorf tubes and elution done using 500µl of NaOH-1%Tris pH 8.0 solution. These were 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 fresh eppendorf tube and boiled at 95-100°C before cooling quickly on ice. The samples were then incubated at -70°C overnight. The following day they were spun at 14000 rpm for 20 minutes, supernatant poured off, and the pellets washed three times in 70% ethanol. Pellets were dried up completely in an eppendorf concentrator 5301™ and suspended in 50µl TE. These were kept at -20°C till needed.
3.10 Polymerase chain reaction

Forty five micro-litres of polymerase chain reaction mixture composed of Taq DNA polymerase Buffer (10µl), 10mM of each dNTP(2µl), 5pmol direct primer, 5pmol reverse primer, Taq 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, annealing at 54°C for 1 minute and elongation at 72°C for 1 minute. This was repeated for 35 cycles, before a further elongation step at 72°C for ten minutes.

Two sets of primers were used in this study. The first set was previously identified by Zhong, et al., (1996) as NV1 5’ CGTGATGCGATAAAGTAGCG 3’ and (3’ CCTCCTTTACATAAGACAAAC 5’), and used in PCR assays utilizing blood. These primers amplify a region 188 bp long of W. bancrofti DNA sequence. The other primers used were coded as DC1 (5’-TTTTGATGGTGTAATAATGCAGC-3’) primer length 23 and (3’-CCTAGTTGTCGTCACCAATCC-5’) length 21, which amplified a 254bp, sequenced by Abassi, et al., (1996). After an initial trial, NV1 set of primers was selected for use in the study.

3.11 Gel electrophoresis

Agarose gels were prepared by dissolving 1.2g Agarose 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 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 ran at 100mV for 50 minutes, and the bands viewed on an UV viewer, (Vilber Lumart™). The gels were subsequently photographed for permanent records.

### 3.12 Purification of PCR products by QIAQUICK KIT™ for hybridization

This was carried out according to manufacturer’s instructions (Qiagen™, USA). The PCR products were purified so as to remove contaminants such as the primer dimers and any unused dNTPs. Briefly, 500µl of the provided buffer PB™ was added to 10µl of the PCR product. The spin Q-column™ was placed in the eppendorf and the diluted sample was added to the Q-column™ and centrifuged for 30-60 seconds at 14000 rpm. The flow-through was discarded and the same tube retained. The pellet was washed twice with 750µl of the provided buffer PE™ by centrifuging for 30-60 seconds. The flow-through was discarded and column spun dry for 60 seconds. A fresh eppendorf was introduced and 50µl of buffer EB™ or SDDW. The purified PCR products were stored frozen till needed.

### 3.13 Titration for Dot Blot radioisotope signals

This titration was performed on 10% samples for quality control as well as to ascertain the capacity of the dot blotting technique to pick low DNA concentrations. Fifteen positive and fifteen negative samples from the PCR products were picked randomly. The positive and the negative controls were included. The samples were
run on Agarose gel to determine bands intensity. The most intense readings on the gels were diluted 1:4 (10μl sample and 30μl TE). Five tubes were set for each sample and serial dilutions carried out in volumes of 20μl.

### 3.14 Dot Blot protocol

The volume of the DNA to be spotted was adjusted to 20μl, before adding 5μl of 4.5M Sodium hydroxide and 25μl of 4.0 M Ammonium acetate to each sample. Using a plastic box, the nitrocellulose paper was soaked in SDDW and 2.0 M Ammonium acetate for at least 10 minutes respectively.

The dot blot apparatus (Bio-Dot®, Bio-rad, USA) was connected to the vacuum pump. Whatman filter and the treated nitrocellulose paper (NCP) were inserted. The samples were neatly spotted while the apparatus was running. The NCP was removed and marked at one corner for orientation. It was air dried and cross-linked (1 minute) in a cross-linking oven or baked in vacuum oven for two hours at 60°C. The NCP was then put in a clean envelope and kept until ready for hybridization.

#### 3.14.1 Hybridization

Pre-hybridization of the dot blot was carried out as described by Sambrook et al., (1989). Briefly, 20ml of hybridization buffer (Appendix 5) was added into the hybridization tubes, carefully, the NCP was introduced into the tube and pre-hybridized for 1-2 hrs at 60°C. Hybridization was done as follows; 10-25ng of Probe (W. bancrofti Plasmid PCR products obtained from Hebrew University) was adjusted to a final volume of 11μl. The hybridization tube was carefully opened and 1ml of hybridization solution which had the $^{35}$S radio-active probe with a final concentration
10^6 CPM/ml of the total hybridization solution. The solution was well mixed and hybridization carried out at 65°C overnight in a Stuart scientific™ hybridization oven. The hybridization solution was removed carefully and 2xSSC -0.1% SDS (washing buffer) (Appendix 5) added into the hybridization tube and let to run for 30 minutes at 60°C. The washing buffer was changed twice and washed for 15 minutes each time. The NCP was air dried for 10-20 minutes before exposing to X-ray film 2 hours at -70°C (Ausubel et al., 1999, Sambrook et al., 1989)

3.14.2 Calculating the amount of 35S for use in hybridization

After labeling the probe, 2 µl of the labeled solution were applied on to a glass-fiber mat in a scintillation vial. The scintillation fluid (2 ml) was added and the counts per minutes (CPM) were read in a scintillation beta- counting machine. The counts were recorded and calculated as follows.

Example:

When the reading was 437 000 CPM and the volume of DNA after purification of the radio-labeled probe is 70 µl. Then, if there were 100% incorporation this would mean (70/2 x 435 000) or 15 225 000 CPM per 25ng (weight of DNA). Approximately this equals 15 x10^6 CPM/µg. Or 15 x10^6 per 70µl, which means approximately, 1.5 x 10^5 per µl. Thus 10^6 CPM /ml. were needed.

Therefore, for 20 ml hybridization solution, 20 x 10^6 CPM was required. Therefore, if 20 µl of probe were used, then it would contain 20 x 1.5 x 10^6 CPM = 30 x 10^6 CPM/ml. Therefore 30 µl of probe was taken for the labelling.
3.15 The sensitivity, specificity, predictive values and accuracy of Tests

Sensitivity and specificity for the two PCR assays (sputum and urine) were performed by 2 x 2 Table method as suggested by Greenhalgh (1997). Similarly positive (PPV) and negative (NPV) predictive values were calculated by the following formulae:

\[
\text{PPV} = \frac{\text{True positive}}{\text{True positive} + \text{False positive}} \times 100
\]

And

\[
\text{NPV} = \frac{\text{True negative}}{\text{True negative} + \text{False negative}} \times 100
\]

Accuracy of the tests was calculated according to the formula below suggested by Greenhalgh (1997).

\[
\text{Accuracy} = \frac{(\text{True positive} + \text{True negative})}{(\text{True positives} + \text{True negatives} + \text{False positive} + \text{False negative})} \times 100
\]

3.16 Data management and analysis

The positive bands in the gel electrophoresis were taken as those appearing at 188bp, where the size of positive control is expected to appear. The negative results were
taken as those samples, which do not show a band at the expected zone of 188bp. The results were recorded along with the sample identification parameters such as name, age, sex etc, in a Microsoft excel spread-sheet for statistical analysis. Using the statistics software statview, the results were subjected to spearman correlation and student t-test to identify any statistical significance between ICT and PCR results.
CHAPTER FOUR

RESULTS

4.1. General samples analysis

Three hundred and four (304) samples of each specimen sputum, urine and blood were obtained for the study (Table 1). One hundred and twenty three were obtained from females, while 181 were from males. The ages of the study group, ranged from 5 - 73 years. The location had five villages: Garashi, Madzayani, Majehazini, Mbaoni, and Sabaki (Appendix 1).

Table 1: Distribution of the study population among the five villages

<table>
<thead>
<tr>
<th>Village</th>
<th>Females</th>
<th>Males</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Garashi</td>
<td>18</td>
<td>21</td>
<td>39</td>
</tr>
<tr>
<td>Madzayani</td>
<td>58</td>
<td>93</td>
<td>151</td>
</tr>
<tr>
<td>Majehazini</td>
<td>24</td>
<td>31</td>
<td>55</td>
</tr>
<tr>
<td>Mbaoni</td>
<td>12</td>
<td>22</td>
<td>34</td>
</tr>
<tr>
<td>Sabaki</td>
<td>14</td>
<td>11</td>
<td>25</td>
</tr>
<tr>
<td>Total</td>
<td>126</td>
<td>178</td>
<td>304</td>
</tr>
</tbody>
</table>

Although the ages ranged from 5 - 73 years, the age group 11 - 20 years had the most persons compared to the other ages (appendix 2). This is due to the fact that there are more persons in this age group than in the other age groups (Government of Kenya
census, 1999) and being young, they were more active and seemed to understand better the reasons for the study.

4.2 Results of microfilaria (MF) - counts by chamber method

Microfilariae were found in 67 samples out of the total 304. The counts ranged from 1 microfilaria to 866 microfilariae per millilitre of whole blood. In table 2, the samples were represented in four groups according to the microfilariae counts in logarithmic factors of 10. Group 1 samples did not have any microfilariae, group 2 had 1 to 10 microfilariae per ml of whole blood, group three 11 to 100 while group 4 ranged from 101-1000.

Table 2: The distribution of microfilaria (MF) counts in the samples

<table>
<thead>
<tr>
<th>Group</th>
<th>Microfilaria counts/ml of blood</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>237</td>
</tr>
<tr>
<td>2</td>
<td>1 – 10</td>
<td>41</td>
</tr>
<tr>
<td>3</td>
<td>11 – 100</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>101 – 866</td>
<td>7</td>
</tr>
</tbody>
</table>

Wuchereria bancrofti infection was determined in the different age groups and it was found that it was highest among the age group 51-60 years with 37.0% positivity rate followed by age group 31-40 (35.5%), and age group 61-73 (31.3%). The lowest positivity rate was in the 5-10 year group with 9.7% (Table 3). Age group 11-20 had
15.7% positivity and age group 41-50 and age group 21-30 had 23.1% and 25% respectively.

<table>
<thead>
<tr>
<th>Age groups (years)</th>
<th>Total number of samples</th>
<th>Number positive by MF counts</th>
<th>Percentage of total MF counts positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>3</td>
<td>9.7</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>19</td>
<td>15.7</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>13</td>
<td>25.0</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>11</td>
<td>35.5</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>6</td>
<td>23.1</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>10</td>
<td>37.0</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>5</td>
<td>31.3</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>67</td>
<td>22.0</td>
</tr>
</tbody>
</table>

Analysis by sex showed that *W. bancrofti* infection in males was 25.8% (46/178) (Table 4) and that of women was 16.7% (Table 5). In males the highest positivity was 61.5% in the age group 51-60 years, followed by 36.8% of and 32.1% of 31-40 year age group and 21-30 year age group respectively. Low positivity was seen in the age groups 61-73 years (30.0%) and 41-50 years (22.2%). The lowest positivity was in
the age group 5-10 years who had 10.5% followed by age group 11-20 years who had 18.8%.

**Table 4: Microfilaria counts in males according to age groups**

<table>
<thead>
<tr>
<th>Age group (Years)</th>
<th>Total number of samples counted</th>
<th>Total number of positive samples</th>
<th>Number of positive males</th>
<th>Percentage positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>19</td>
<td>2</td>
<td>10.5</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>80</td>
<td>15</td>
<td>18.8</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>28</td>
<td>9</td>
<td>32.1</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>19</td>
<td>7</td>
<td>36.8</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>9</td>
<td>2</td>
<td>22.2</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>13</td>
<td>8</td>
<td>61.5</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>10</td>
<td>3</td>
<td>30.0</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>126</td>
<td>49</td>
<td>38.9</td>
</tr>
</tbody>
</table>

Analysis of the samples from women showed the highest positivity among age groups 31-40 and 61-73 years, who had 33.3%. These two groups were followed by age groups 41-50 (23.5%), 21-30 (16.7%) and 51-60 (14.3%). The lowest positivity was in the age group 5-10 (8.3%) followed by age group 11-20 years who had 9.8% positivity.
### Table 5: Microfilaria counts in females according to age groups

<table>
<thead>
<tr>
<th>Age group (Years)</th>
<th>Total number of samples</th>
<th>Total number of positive males samples</th>
<th>Number of positive Females</th>
<th>Percentage positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>12</td>
<td>1</td>
<td>8.3</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>41</td>
<td>4</td>
<td>9.8</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>24</td>
<td>4</td>
<td>16.7</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>12</td>
<td>4</td>
<td>33.3</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>17</td>
<td>4</td>
<td>23.5</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>14</td>
<td>2</td>
<td>14.3</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>6</td>
<td>2</td>
<td>33.3</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>178</td>
<td>46</td>
<td>25.8</td>
</tr>
</tbody>
</table>

**4.3. Immunochromatographic test (ICT) results**

A total of 119 out of 304 samples were found positive by ICT (Table 6). This constituted 38.8% of the test samples with the highest number of positive cases among the 61-73 age group who had 62.5% positivity. Positive cases among the age groups 51-60, 31-40, and 41-50 years, were 51.9%, 51.6% and 50.0% respectively. Age groups 11-20 and 21-30 had a positivity rate of 33.9% and 34.6% respectively. The lowest positivity was found among the 5-10 year age group who had 22.6%.
### Table 6: Immunochromatographic test (ICT) results of the total test samples

<table>
<thead>
<tr>
<th>Age groups (years)</th>
<th>Total number of samples in each group</th>
<th>Number positive by ICT</th>
<th>Percentage of total ICT</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>7</td>
<td>22.6</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>41</td>
<td>33.9</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>18</td>
<td>34.6</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>16</td>
<td>51.6</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>13</td>
<td>50.0</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>14</td>
<td>51.9</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>10</td>
<td>62.5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>304</strong></td>
<td><strong>119</strong></td>
<td><strong>39.1</strong></td>
</tr>
</tbody>
</table>

There were 178 male samples among the total 304 samples examined, constituting 58.6%. Out of the 178 male population 71 (39.9%) had positive results by ICT (Table 7). The highest positivity was among the 51-60 year age group, who had 76.9% (10/13) with the lowest being the 5-10 year age group showing a positivity of 21.1% (4/19). Age groups 61-73 years, 41-50 years, 31-40 years and 21-30 years had 60.0%, 55.6%, 52.6% and 42.9%, respectively. Twenty four samples out of 80 males (30.0%) samples were ICT positive in the age group 11-20 years. This was the second lowest positivity among the male age groups.
Table 7: Immunochromatographic test in males according to age groups

<table>
<thead>
<tr>
<th>Age group</th>
<th>Total number of samples</th>
<th>Number of males</th>
<th>Number of Positive males</th>
<th>Percentage positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>19</td>
<td>4</td>
<td>21.1</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>80</td>
<td>24</td>
<td>30.0</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>28</td>
<td>12</td>
<td>42.9</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>19</td>
<td>10</td>
<td>52.6</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>9</td>
<td>5</td>
<td>55.6</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>13</td>
<td>10</td>
<td>76.9</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>10</td>
<td>6</td>
<td>60.0</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>178</td>
<td>71</td>
<td>39.9</td>
</tr>
</tbody>
</table>

*Wuchereria bancrofti* infection among females was 38.9% (49/126) (Table 8) by ICT tests. The highest positivity was among the 61-73 year age group with 66.7% (4/6) compared with the least positive 25.0% among the 5-10 (3/12) and 21-30 (6/24) year age groups. Age groups 31-40, 41-50 and 11-20 had 50.0%, 47.1% and 43.9% ICT positivity respectively. Age group 51-60 years had four samples out of a total of 14 samples collected from women showing ICT positivity. This was the second lowest positivity at 28.6%. 
Table 8: Immunochromatographic test in females according to age groups

<table>
<thead>
<tr>
<th>Age group</th>
<th>Total number of samples</th>
<th>Total number of females</th>
<th>Number of Positive Females</th>
<th>Percentage of positive females/females</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>12</td>
<td>3</td>
<td>25.0</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>41</td>
<td>18</td>
<td>43.9</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>24</td>
<td>6</td>
<td>25.0</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>12</td>
<td>6</td>
<td>50.0</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>17</td>
<td>8</td>
<td>47.1</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>14</td>
<td>4</td>
<td>28.6</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>6</td>
<td>4</td>
<td>66.7</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>126</td>
<td>49</td>
<td>38.9</td>
</tr>
</tbody>
</table>
FIGURE 6: Typical 2% Agarose gel electrophoresis results of the PCR products from sputum and urine.

Lanes 1-7 Positive samples showing typical 188 bp band of W. bancrofti DNA
Lanes 8-11 Negative samples (note absence of 188 bp band)
Lane 12 Positive control
Lane 13 100bp ladder molecular marker.

4.4. Polymerase chain reaction PCR assays results

Initial trials on optimization of the PCR assays, established that the NVI primers that amplified a band which was 188bp seemed to have more intense bands than DC1
primers that amplified a 254bp band. Other trials on the appropriate method for DNA extraction from urine settled for a method that included a filtration stage with nitrocellulose paper. Typical bands of PCR products from either sputum or urine are shown in figure 6.

Table 9: Polymerase chain reaction (PCR) assay for sputum in all samples according to age groups

<table>
<thead>
<tr>
<th>Age group</th>
<th>Total number of samples</th>
<th>Number Positive samples</th>
<th>Percentage Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>9</td>
<td>29.0</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>43</td>
<td>35.5</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>21</td>
<td>40.4</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>18</td>
<td>58.1</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>15</td>
<td>57.7</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>14</td>
<td>51.9</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>10</td>
<td>62.5</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>130</td>
<td>42.8</td>
</tr>
</tbody>
</table>

Wuchereria bancrofti infection detected by PCR sputum assay was 42.8% (130) for all the age groups (Table 9). Among the age group 61-73 years there was a high
number of positive cases 62.5% (10/16) followed by the age group 31-40 with 58.1% (18/31), age group 41-50 with 57.7% and age group 51-60 with 51.9%. Out of 52 samples in the age group 21-30, there were 21 (40.4%) sputum PCR positive samples. Forty three samples (35.5%) were sputum PCR positive in the age group 11-20 years, compared with 29.0% (9/31) in the age group 5-10 years, which had the lowest positivity (Figure 7).

Figure 7: Percentage of positivity by sex and age in sputum PCR assay. A=5-10; B=11-20; C=21-30; D=31-40; E=41-50; F=51-60; G=61-73 years.
Table 10: Polymerase Chain Reaction (PCR) assay for sputum in males according to age groups.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Total number of samples</th>
<th>Total number of males</th>
<th>Number of Positive males</th>
<th>Percentage Positive male/male</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>19</td>
<td>6</td>
<td>31.6</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>80</td>
<td>23</td>
<td>28.8</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>28</td>
<td>13</td>
<td>46.4</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>19</td>
<td>11</td>
<td>57.9</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>9</td>
<td>5</td>
<td>55.6</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>13</td>
<td>9</td>
<td>69.2</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>10</td>
<td>6</td>
<td>60.0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>304</strong></td>
<td><strong>178</strong></td>
<td><strong>73</strong></td>
<td><strong>41.0</strong></td>
</tr>
</tbody>
</table>

Seventy three males (41.0%) were sputum PCR positive out of a total of 178 males. There was high PCR positivity among the age group 51-60 years with 69.2% (9/13) (Table 10). The other age groups; 61-73, 31-40, 41-50, and 21-30 followed with 60.0%, 57.9%, 55.6% and 46.4%, respectively. The lowest number of sputum PCR positive cases occurred in 11-20 year age group with 28.8% (23/80) followed by age group 11-20 years with 31.6%.
Table 11: Polymerase Chain (PCR) Assay for sputum in females according to age groups

<table>
<thead>
<tr>
<th>Age group</th>
<th>No. Total samples</th>
<th>No. Total females</th>
<th>Positive females</th>
<th>Percentage Positive female/female</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>12</td>
<td>3</td>
<td>25.0</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>41</td>
<td>20</td>
<td>48.8</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>24</td>
<td>8</td>
<td>33.3</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>12</td>
<td>7</td>
<td>58.3</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>17</td>
<td>10</td>
<td>58.8</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>14</td>
<td>5</td>
<td>35.7</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>6</td>
<td>4</td>
<td>66.7</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>126</td>
<td>57</td>
<td>45.2</td>
</tr>
</tbody>
</table>

Females positivity in the PCR assay for sputum was high 66.7% (4/6) among the 61-73 year age group (Table 11) compared with 5-10 year group which was the lowest at 25.0% (3/12).
Table 12: Polymerase Chain Reaction (PCR) assay for urine in all samples according to age groups

<table>
<thead>
<tr>
<th>Age group</th>
<th>Total number of samples</th>
<th>Total number of Positive samples</th>
<th>Percentage of Positive males/total males</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>7</td>
<td>22.6</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>38</td>
<td>31.4</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>18</td>
<td>34.6</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>13</td>
<td>41.9</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>12</td>
<td>46.2</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>14</td>
<td>51.9</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>8</td>
<td>50.0</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>110</td>
<td>36.2</td>
</tr>
</tbody>
</table>

Polymerase chain reaction assay for urine had a total of 36.2% (110/304) positivity among the examined samples (Table 12). The lowest positivity in urine samples was 22.6% (7/31) in the 5-10 year age group. The positivity increased gradually through age groups 11-20 (31.4%), 21-30 (34.6%), 31-40 (41.9%) and 41-50 (46.2%). The highest positivity was in the 51-60 year age group with 51.6% (14/27), with only 1.6% above the positivity observed in the 61-73 years age group which had 50% (8/16).
Table 13: Polymerase Chain Reaction (PCR) assay of urine in males according to age groups

<table>
<thead>
<tr>
<th>Age group</th>
<th>Total number of samples</th>
<th>Number of total males</th>
<th>Number of positive males</th>
<th>Percentage of positive males/males</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>19</td>
<td>4</td>
<td>21.1</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>80</td>
<td>21</td>
<td>26.3</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>28</td>
<td>10</td>
<td>35.7</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>19</td>
<td>7</td>
<td>36.8</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>9</td>
<td>4</td>
<td>44.4</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>13</td>
<td>10</td>
<td>76.9</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>10</td>
<td>4</td>
<td>40.0</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>178</td>
<td>60</td>
<td>39.7</td>
</tr>
</tbody>
</table>

Sixty males out of 178 male samples were *W. bancrofti* infected, constituting 33.7% of the total male PCR assay for urine results (Table 13). The highest positivity was among the 51-60 year age group who had 76.9% (10/13) positivity. This was 32.5% higher than the age group 41-50 (44.4%) which followed. The rest of the age groups
had 40% (61-73), 36.8% (31-40), 35.7% (21-30), 26.3% (11-20) and 21.1% in age group 5-10 years.

Female positivity by PCR assay for urine was 39.7% (50/126) (Table 14), with the highest being 66.7% (4/6) in the 61-73 year age group, and the lowest being 25.0% (3/12) in the 5-10 year age group. Age group 51-60 had 28.6% positivity followed by 21-30 (33.3%), 11-20 (41.5%), 41-50 (47.1%), and 31-40 (50.0%)

Table 14: Polymerase Chain Reaction (PCR) assay of urine in females according to age groups.

<table>
<thead>
<tr>
<th>Age group</th>
<th>Number of total samples</th>
<th>Number of total females</th>
<th>Number of Positive Females</th>
<th>Percentage of positive females/females</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-10</td>
<td>31</td>
<td>12</td>
<td>3</td>
<td>25.0</td>
</tr>
<tr>
<td>11-20</td>
<td>121</td>
<td>41</td>
<td>17</td>
<td>41.5</td>
</tr>
<tr>
<td>21-30</td>
<td>52</td>
<td>24</td>
<td>8</td>
<td>33.3</td>
</tr>
<tr>
<td>31-40</td>
<td>31</td>
<td>12</td>
<td>6</td>
<td>50.0</td>
</tr>
<tr>
<td>41-50</td>
<td>26</td>
<td>17</td>
<td>8</td>
<td>47.1</td>
</tr>
<tr>
<td>51-60</td>
<td>27</td>
<td>14</td>
<td>4</td>
<td>28.6</td>
</tr>
<tr>
<td>61-73</td>
<td>16</td>
<td>6</td>
<td>4</td>
<td>66.7</td>
</tr>
<tr>
<td>Total</td>
<td>304</td>
<td>126</td>
<td>50</td>
<td>39.7</td>
</tr>
</tbody>
</table>
4.5 Validation of the PCR assays

Sensitivity and specificity for the two PCR assays (sputum and urine) were performed as suggested by Greenhalgh (1997). Sensitivity was defined as the ability of the PCR assays to identify *W. bancrofti* DNA in ICT positive samples. The sensitivity and specificity of PCR assay for sputum was found to be 97.5% and 92.4%, respectively, where, the positively identified samples were 116 out of 119 ICT positive samples. There were 3 false negative samples. One hundred and seventy four samples were negative by PCR assay for sputum. False positives were 14 (Table 15).

4.5.1 Calculation of Sensitivity and Specificity of the tests

The formula of Greenhalgh (1997) was used in both calculations.

I) Sensitivity of PCR assay for sputum taking ICT as standard.

<table>
<thead>
<tr>
<th></th>
<th>ICT positive</th>
<th>ICT negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum positive</td>
<td>116</td>
<td>14</td>
<td>130</td>
</tr>
<tr>
<td>Sputum negative</td>
<td>3</td>
<td>171</td>
<td>174</td>
</tr>
<tr>
<td>Total</td>
<td>119</td>
<td>185</td>
<td>304</td>
</tr>
</tbody>
</table>

\[
\text{Sensitivity} = \frac{116}{116 + 3} \times 100 = 97.5\%
\]
II) Specificity of PCR assay for sputum.

\[
\text{Specificity} = \frac{171}{171 + 14} \times 100 = 92.4\%
\]

4.5.2 Calculation of Predictive values

Predictive values were calculated using the formula by Greenhalgh (1997).

(I) Positive predictive value PPV

\[
\frac{116}{116 + 14} = 89.2\%
\]

(II) Negative predictive value NPV

\[
\frac{171}{171 + 3} = 98.3\%
\]

4.5.3 Accuracy of the PCR assays

This was calculated as the true positives and true negatives as a proportion of all results (Greenhalgh, 1997).

\[
\frac{116 + 171}{116 + 171 + 14 + 3} \times 100 = 94.4\%
\]

Similarly, accuracy in PCR for urine was 95.1%
Sensitivity and specificity of PCR assay urine were 96.1% and 94.5% respectively, where the positively identified samples within the ICT positives were 99. Four samples were false negative. The negative samples among the ICT negatives were 11 (Table 15).

Table 15: Validation data for PCR assays

<table>
<thead>
<tr>
<th></th>
<th>TRUE</th>
<th>TRUE</th>
<th>FALSE</th>
<th>FALSE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>POSITIVE</strong></td>
<td>(ICT +)</td>
<td>(ICT -)</td>
<td>(ICT -)</td>
<td>(ICT +)</td>
</tr>
<tr>
<td>PCR (sputum)</td>
<td>116</td>
<td>171</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>PCR (urine)</td>
<td>99</td>
<td>190</td>
<td>11</td>
<td>4</td>
</tr>
</tbody>
</table>

Positive predictive value (PPV) was defined as the ability of a positive PCR assay test to indicate that the *W. bancrofti* DNA is present in a sample. Consequently, the Negative predictive value (NPV) was defined as the ability of a negative PCR assay to indicate *W. bancrofti* DNA was absent in the negative sample (Greenhalgh, 1997).

Thus the PPV for PCR assay for sputum was 89.2% and NPV 98.3%. As for PCR assay for urine, PPV was 90.0% and NPV 97.9%.
Table 16: Lymphatic filariasis positivity obtained from the different diagnostic tests used in the study.

<table>
<thead>
<tr>
<th>Lymphatic filariasis</th>
<th>ICT</th>
<th>MF counts</th>
<th>PCR Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine</td>
<td>sputum</td>
<td></td>
</tr>
<tr>
<td>Positivity</td>
<td>38.8%</td>
<td>22.0%</td>
<td>36.2%</td>
</tr>
</tbody>
</table>

ICT= Immunochromatographic test, MF= microfilaria

Accuracy of the PCR assays was defined as the proportion of all tests that have given the correct result as compared with ICT. This was calculated as the true positives and true negatives as a proportion of all results. The accuracy of PCR assay for sputum was 94.4% while that of PCR assay for urine was 95.1%
Table 17: Sensitivity and Specificity of PCR assays.

<table>
<thead>
<tr>
<th>PCR assay</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>97.4</td>
<td>98.3</td>
<td>93.5</td>
<td>86.2</td>
<td>94.4</td>
</tr>
<tr>
<td>Urine</td>
<td>96.1</td>
<td>94.5</td>
<td>90.0</td>
<td>89.2</td>
<td>95.1</td>
</tr>
</tbody>
</table>

PPV = Positive predictive value; NPV = Negative predictive value

Table 18: Correlation of positivity in various tests used in the study

<table>
<thead>
<tr>
<th></th>
<th>MF counts</th>
<th>ICT</th>
<th>PCR assay</th>
<th>PCR assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>MF counts</td>
<td>67</td>
<td>65</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>ICT</td>
<td>65</td>
<td>119</td>
<td>116</td>
<td>99</td>
</tr>
<tr>
<td>PCR assay-sputum</td>
<td>67</td>
<td>116</td>
<td>130</td>
<td>108</td>
</tr>
<tr>
<td>PCR assay-urine</td>
<td>67</td>
<td>108</td>
<td>108</td>
<td>110</td>
</tr>
</tbody>
</table>

4.6 Quality control by dot blot hybridization

Thirty samples, constituting about 9.87% (30/304) of the study group were selected by picking randomly fifteen positive and fifteen negative PCR assay samples from both urine and sputum. Positive results were determined by appearance of black dots on the photographic film while negative results showed no dots (Figure 8). These representative results indicated that all the 15 PCR positive samples had the specific
*W. bancrofti* DNA, while the negative samples did not have the specific *W. bancrofti* DNA.

FIGURE 8: Dot blot results from 30 randomly selected samples
Lane P = Positive control, 2-4 = Positive samples, N = Negative control
Figure 9: Lymphatic filariasis tests compared to PCR sputum assay
CHAPTER FIVE

DISCUSSION

Although *Wuchereria bancrofti* infection is not considered to be a cause of mortality, the filarial morbidity is a devastating and crippling affliction that causes social and economic hardships to affected communities. Currently 128 million people living in the weak economies of the tropics are infected (WHO 1998), thus reducing in the economic productiveness, and stretching further the available resources in maintaining the sick. Over 1.1 billion people or every sixth person in the world, are at risk of infection with lymphatic filariasis (WHO 1998). Due to this, and the recent research advances in treatment and control, lymphatic filariasis has been identified by world health organization for elimination (WHO, 1998).

The current study employed four diagnostic tests to establish *W. bancrofti* infection prevalence in 304 samples obtained from Malindi district, Kenya, a known endemic region for lymphatic filariasis. By using microscopy, where microfilaria were enumerated using the chamber method, 22.0% (67/304) of the samples were found to be positive. This correlated well with the findings of Wijers, (1977), who found 20.0% infection rate in the adjacent region of Kwale district with prevalence in males (54.6%) being higher than females (43.4%). Although data obtained in the study did not indicate any statistical differences between sexes and ages, overall results show more males to be infected (68.7%) compared to females (31.3%), findings supported by other reports (Wijers, 1977). Other than report of Wijers, (1977), discussed here, Weerasooriya (2003) found 62.2% males positivity against 49.1% in females from Sri
Lanka. Other available data is also indicative of similar results (Brabin, 1990, World Bank, 1993). Probably this is due to the fact that males tend to have more outdoor activities than females and may have greater interaction with peak mosquito biting time.

Continued exposure to the infectious mosquitoes (Manson-Barr and Apted, 1984), may explain why the data shows a gradual rise in positivity from the young age groups towards the older age groups (Figure 7). This is also consistent with other findings. For instance, Weerasooriya (2003) found positivity of 24.5% in ages 5 years and below, and 70% in people above 50 years. Microscopy gave the least positivity (Appendix 1) compared to all the tests in the present study (Table 16). This is consistent with other findings such as those of Siridewa, (1996) who constantly found PCR showing higher positivity than microscopy, when peripheral blood, hydrocoele fluid and mosquito’s samples were used in a comparative study in Sri Lanka.

Other workers (Wamae, 1994, Lucena et al, 1996, Abassi et al, 1999, Weil et al 1996, Njenga and Wamae, 2001) have also found microscopy to be least sensitive. Microscopy has been the traditional method of diagnosis of lymphatic filariasis. It is fairly cheap and specific as the parasite is displayed under a microscope, and identification of the species is possible through staining (Manson-Barr and Apted, 1984). Unfortunately for effective microscopy, blood has to be collected at night between 2200 and 0200 hrs, when the microfilaria of this nocturnal parasite can be found in the blood (Manson-Barr and Aped, 1984). This poses various problems, one of which is the need to have trained medical teams to collect blood samples. The communities in the endemic regions are sensitive to being bled, especially at midnight
There are also problems of transporting medical teams in terms of cost, security and other logistics (Njenga and Wamae, 2001). The low sensitivity of microscopy, coupled with these problems presents microscopy as an unattractive method for diagnosis of lymphatic filariasis particularly on a large scale or for epidemiological surveys.

Immunochromatographic test (ICT) is a rapid card test, which can use day blood for diagnosis of lymphatic filariasis. In this study, 38.8% (119/304) of samples were positive by ICT using venous blood (Table 16). Prevalence studies have not been carried out in the study area. However, a study done in Muhaka, Kwale District, in the south coast, by Njenga and Wamae, (2001) showed an infection rate of 37.3% by ICT, indicating that prevalence in Malindi district may be similar to that of Kwale district. Immunochromatographic test (ICT) detects the circulating filarial antigen, (CFA) (Weil et al, 1997). The sensitivity of detecting lymphatic filariasis cases without, or with low microfilaremia by antigen is low (Weil et al, 1997, Lucena et al, 1998, Abbasi et al, 1999, Audrey et al, 2000, Pani et al, 2000). There is also inconsistence in the performance of the ICT. In the study carried out by Njenga and Wamae, (2001), it was shown that ICT could react variably, depending on whether serum or whole blood was used. Similarly there were differences when venous or capillary blood was used. In another study by Pani et al, (2000) the ICT failed to identify 67% of lymphatic filariasis with microfilaria counts of less than 10 per ml. Audrey et al, (2000) found ICT false positivity in 70% lymphatic filariasis patients 3 years after treatment, suggesting an alternative method in surveillance post elimination of LF.
Mass collection of blood in regions with shortages of human expertise and high endemicity of blood borne diseases is problematic. Apart from unco-operating communities (Mwobobia *et al*., 2000), and the lack of trained manpower, there exists risk of HIV/AIDS infection (Marcus, 1988). In Kenya prevalence rates of HIV/AIDS stands at 10% (NASCPO report, 2003).

Dissayanake *et al* (2000) also suggested use of other body fluids after he evaluated blood PCR assays. Other diseases are common, such as hepatitis B (Miller *et al*., 1998) and obviously pose a risk to health workers (Koff *et al*., 1977). In seeking a non-invasive approach for diagnosis of *W. bancrofti* infection, the following two PCR assays utilizing sputum and urine were adapted and tried.

The two PCR assays employed in this study are both sensitive and specific with values above 94% (Table 17). The specificity of the PCR assays was confirmed by dot blot hybridization. The dot blot hybridization has a high sensitivity than PCR (Ausubel *et al*., 1999). The hybridization test is not routinely employed due to its application of radioactivity, but has been used as a confirmatory test (Abbasi *et al*., 1999). All the randomly selected positive samples (15) were confirmed by dot blot hybridization as positive (Sensitivity = 100%) and all negative samples (15) were also confirmed to be negative by dot blot hybridization (Specificity = 100%).

In the current study, sputum PCR assay for sputum had 42.8% (130/304) positivity which was the highest in the four methods tested (Table 16). There were 14 positive samples by PCR, but negative by ICT (Table 15). All the 14 samples had 0-10 microfilaria counts per ml, for which ICT may have missed because it has low
sensitivity (Pani et al, 2000) or because venous blood was used (Njenga and Wamae, 2001). Three samples were false negatives (Table 15); they may have been previously treated cases that ICT may have picked. Immunochromatographic test is known to continue being positive several years after treatment for reasons not clearly understood (Audrey et al, 2000). Working with Haitian patients Audrey et al., (2000) found 70% of samples positive 3 years post treatment, but were microfilaria negative.

Absence of macromolecules in urine is well known, but nonetheless, W. bancrofti DNA has been found in urine as demonstrated by Lucena et al, (1998) and the current study. This suggests the excretion of parasite DNA in the absence of obvious glomerulopathy. This is also true in cases of other infectious diseases such as Legionella pneumonia (Murdoch et al, 1996) and Lyme disease (Bayer, et al., 1996). Van der Hel et al., (2002) working on quality and quantity of DNA isolated from urine could obtain and demonstrate DNA of 198 - 547bp by PCR. Other workers such as Reddy, (1984) and Lutsch et al., (1988) have used urine in diagnosis of W. bancrofti. The PCR assay for urine was found to have less positivity (36.2%) than both PCR assay sputum (42.8%) and ICT (38.8%) but higher than microscopy (22.0%). Although there was no statistical difference in these results, the assay was unable to pick 18 samples (Table 17) when compared to sputum assay, whereas sputum assay failed to pick 2 samples that were positive by urine assay. The low positivity by urine could be explained by reduced availability of DNA and times of collection for urine. Lucena et al., (1998) demonstrated that there was high positivity in urine PCR assay (59.7%) when morning urine samples were used against those of
daytime collection (41.8%). It could also be as a result of failure of ethidium bromide staining, which was found inferior in cases of low DNA concentrations (Van der Hel et al., 2002).

There are some obvious advantages of the two PCR assays used in this study. The assays were both sensitive and specific (Table 17). A test is valid if it detects most people with the target disorder (high sensitivity) and excludes most people without the disorder (high specificity), and if a positive test usually indicates that the disorder is present (high positive predictive value) (Greenhalgh, 1997). The PCR assays in this study have demonstrated high sensitivity and specificity as well as high accuracy values for both sputum and urine.

By using PCR assays that utilize sputum and urine, there are reduced risks of infection with the blood borne infectious diseases such as HIV/AIDS and Hepatitis. Recent studies show an increased prevalence in HIV/AIDS and tuberculosis in Kenya (NASCOP report, 2003, Odhiambo et al, 1999). More importantly the study further shows that 0.3% of health workers may seroconvert to HIV/AIDS positive status (Marcus, 1988). Infection of hepatitis has been reported to be as high as 5-10% (Sepkowitze, 1996). Data on National hepatitis prevalence in Kenya is lacking, though it is suspected to be high (Miller et al, 1998). It is eminent that the health workers are at risk of hepatitis infection (Mosley, 1975, Koff et al., 1977) and the use of non-blood samples may reduce this risk.

Community co-operation is very essential in disease elimination efforts, such as the one underway for lymphatic filariasis. By use of non-invasive samples collection, the
communal participation can be enhanced. It is easy to obtain samples such as urine even from young children as reported by Weerasooriya et al., (2003). Where trained manpower is not available, local people can be trained to get samples and transport them to a central laboratory. Involving local communities to collect samples can ease some of the logistical problems encountered by visiting medical teams, and in access areas that cannot be reached by vehicles (Lucena et al., 1998). It will also increase their awareness and overall participation in such a study.

By having local people participating in samples collection, and using the sensitive and specific molecular techniques in a central laboratory, a winning combination would be reached with far reaching results in elimination of the disease.

The application of molecular methods, and in particular the PCR assays has been regarded as unaffordable in lymphatic filariasis (Wamae, 1994). But even the poor deserve quality results, and if the 128 million patients (WHO 1998) could stop being dependent and be able to contribute to the economies, then the cost of PCR assays would be a dollar well spent. Further, in the current globalization effects, the third world needs to catch up with the rest of the world in terms of modern technology.

Several researchers have suggested pooling of samples in mass diagnosis and epidemiological studies. Abbasi et al., (1999) was able to pool 14 negative Israeli samples with one positive sample and was able to detect the \textit{W. bancrofti} DNA. Similarly Ramzy et al., (2003), Chateau, et al., (1994) and Katholi et al., (1995) worked with pooled vectors and blood with similar results. Pooling of samples by house hold, would reduce costs of PCR assays probably by up to 90% as average house holds in Malindi have on average 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. Consequently this would increase the chances of positivity among the households with *W. bancrofti* infections if the samples were pooled. The positive households could then benefit by treatment of all members as recommended by WHO (1998) or individual members could be analysed to identify the actual patients.
CHAPTER SIX
CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions
The present study examined *W. bancrofti* infections in Malindi district, Kenya. Polymerase chain reaction assays using both sputum and urine were used in the study. The study also compared the results obtained from these assays to ICT and microfilariae counts. The study therefore concludes that:

1. The prevalence of *W. bancrofti* infection in the samples was 42.8% using PCR assay for sputum and 36.2% using PCR assay for urine.
2. The PCR assays used in this study had sensitivity of above 94.0% specificity of above 96.0% and accuracy of above 95.0%. The assays employed samples that can be collected easily by non-invasive methods.
3. The PCR assays used can complement other methods used for epidemiology, mass diagnosis and surveillance of lymphatic filariasis post eradication phase.

6.2 Recommendations
From the outcome of this study, PCR assays that employ the use of non invasive sample collection are recommended for use in mass diagnosis, epidemiology and surveillance of lymphatic filariasis.

The study also recommends that more research be carried out to optimize the use of PCR assay for urine as recent figures indicate increase in tuberculosis spread by droplet infection, hence the need of reduction of sputum use as a sample.
The pooling of samples per household and other methods that could be useful in cost reduction of performing PCR assays should be investigated.

Research on the prevalence of tropical pulmonary eosinophilia (TPE) should be done so as to establish whether the elevated positivity in PCR assay for sputum is an indication of the disease.

A survey to establish the extent of *W. bancrofti* infections in the rest of Kenya should be carried out so that elimination program can be expanded to cover all the affected areas.
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statistics.


Appendix 1: The study area showing the villages where the study samples were obtained.
Appendix 2: Age frequency in the study population showing high population in the age group 10-20 years
Appendix 3: Chest X-ray of a patient suffering from tropical pulmonary eosinophilia TPE. Adapted from WHO 2000
Appendix 4: Elephantiasis showing deformation on one leg. Adapted from WHO 2000
Appendix 5

PREPARATION OF REAGENTS

(1) **2M Ammonium acetate 1L**

154g of Ammonium acetate were dissolved in 1000ml of sterile double distilled water.

(2) **4m Ammonium acetate 100ML**

30.8g Ammonium acetate was dissolved in 100ml of sterile double distilled water.

(3) **4.5m Sodium hydroxide 100ML**

18.0g of sodium hydroxide was dissolved in 100ml of sterile double distilled water.

(4) **20x Sodium citrate- Sodium chloride (SSC) 1L**

Sodium citrate 0.3M 88.2g
Sodium chloride 3M 175.3g

The above were dissolved in 1000ml sterile double distilled water.

(5) **Denhardts’ solution 50x**

The following reagents were weighed and dissolved in 250ml sterile double distilled water.

2.5g Ficoll 400
2.5g PVP [Polyvinyl pyrrolidine]
2.5g BSA [Bovine serum albumin]

Add SDDW up to 250ml

This was then aliquoted in 50ml volumes and kept frozen at -20 °C.
(6) **Salmon Sperm (DNA) 10mg/ml**

After weighing 50mg salmon sperm, in a 15 ml tube, 5ml of sterile double distilled water was added. It was vortexed until dissolved. Alternatively, it was boiled for 10 minutes. This was then labeled and kept frozen at −20°C.

(7) **10% Sodium Dodecyl Sulphate (SDS)**

50g of sodium dodecyl sulphate was weighed and dissolved in 500ml sterile double distilled water.

(8) **Hybridization buffer**

The following reagents were added into a 50ml tube,

- Stock 20x SSC: 6ml
- Stock 50x Denhardt’s: 2ml
- Stock 100μg/ml salmon sperm DNA: 200μl
- Stock 10% SDS: 200μl

Sterile double distilled water was added up to 20ml. Each hybridization tube used 20ml of hybridization buffer.

(9) **Labeling mix [Hybridization solution]**

- PCR – product (0.1ng): 10μl
- Labeling mix solution™ [Biological industries]: 4μl
- 35S dCTP [3000 ci/mmol]: 5μl

Reagents were carefully added and vortexed.

(10) **0.5% EDTA**

**Weigh 5g of EDTA and dissolve in 1L DDW**

(11) **0.2% EDTA**

**Weigh 0.2g EDTA and dissolve in 100ml DDW**
(12) **Washing buffer 1L (2x SSC, 0.1% SDS)**

100ml of stock x20 SSC
10ml of stock 10% SDS
Sterile double distilled water was added up to 1000ml

(13) **1M Tris acetate (TAE) buffer**

242gms Tris base
57.1 ml Glacial acetic acid
100ml 0.5M EDTA pH8.0
Add DDW to 1L

(14) **0.5M EDTA 1L**

20gms NaOH
372gms EDTA
Add 800ml of DDW
Adjust pH to 8.0
Make up to 1L

(15) **TE Buffer (Tris –EDTA) pH 8.0**

10mM Tris HCl, pH 8.0
1mM EDTA pH 8.0

(16) **1M Tris-HCl pH 8.0, 1L**

Tris 121.1g
HCl conc., 42 ml
Adjust pH to 8.0

(17) **EDTA 1mM**

0.5M EDTA pH 8.0 2ml
DDW 1000ml
Adjust pH to 8.0

(18) **PCR master mix**

Deoxy-Nucleoside Tri-phosphates (dNTP)

<table>
<thead>
<tr>
<th>Name</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine-dNTP</td>
<td>(dATP) 10mM</td>
</tr>
<tr>
<td>Cytidine-dNTP</td>
<td>(dCTP) 10mM</td>
</tr>
<tr>
<td>Guanosine –Dntp</td>
<td>(dGTP) 10mM</td>
</tr>
<tr>
<td>Thymidine-dNTP</td>
<td>(dTTP) 10mM</td>
</tr>
</tbody>
</table>
/ Master mix continued

10x Taq-Polymerase Buffer pH 8.0
Primers 10µl
Taq-polymerase 2µl

For use,
Add 90µl of the mix to each tube
Add 10µl of DNA sample
Appendix 6

INFORMED CONSENT FORM

DIAGNOSIS OF *Wuchereria bancrofti* INFECTION USING POLYMERASE CHAIN REACTION OF SPUTUM AND URINE

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 Malindi district, Kenya, the infection of *Wuchereria bancrofti* (lymphatic filariasis) using sputum-PCR assay, urine PCR assay, ICT and microscopy. The study will compare the sensitivity, specificity and logistic friendliness of the two PCR assays. 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 trained clinician and with the participation of local health workers. You will then be asked to perform a short physical exercise after which you will give a sample of sputum. You will also be asked to donate a urine and blood sample. These samples will be used for the tests indicated below.
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) **Alternative treatments**

   You will be informed of other methods of treatment if any.

4) **Confidentiality of the records**

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

5) **PARTICIPANT’S* OR GUARDIAN’S* SIGNATURE.**

   *Tick the appropriate.*