MOLECULAR CHARACTERIZATION OF *WUCHERERIA BANCROFTI* IN MOSQUITOES OF PANGANI DISTRICT, NORTH EASTERN TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF THE SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

Wuchereria bancrofti is the most widely distributed of the three nematodes known to cause lymphatic filariasis, the other two being *Brugia malayi* and *B. timori*. The present study was carried out to investigate strains of *W. bancrofti* in mosquito vectors responsible for lymphatic filariasis transmission in Pangani district, north-eastern Tanzania. In addition, the vector abundance and vector infection rates were investigated. The presence of *W. bancrofti* in mosquitoes was determined by polymerase chain reaction (PCR) using primers NV1 and NV2 while Poolscreen2 software was used to determine *W. bancrofti* infection rate in mosquitoes. A total of 951 mosquitoes were collected in five randomly selected villages of Pangani district including Bweni, Madanga, Meka, Msaraza and Pangani West. Out of 951 collected mosquitoes, the majority were *Culex quinquefasciatus* (99.36%) followed by *Anopheles gambiae* (0.32%) and other *Culex* species (0.32%). The *W. bancrofti* vector infection rate in the present study was found to be 35.1%, indicating that there may be positive individuals in Pangani district. Phylogenetic analysis of *Ssp 1*repeat region sequence of *W. bancrofti* obtained in the study clustered the parasite into a distinct group compared with other *W. bancrofti*. In addition, the *W. bancrofti* sequences in Pangani district were not, 100% identical but genetically related. Further studies, using alternate typing methods, are however required for an in-depth understanding of strains that respond more slowly to drugs or strains that demonstrate greater fecundity. The information would enhance strategy development regarding the impact of mass drug administration (MDA), such as how long to run an MDA program and the optimal size of the human population treatment unit.

DECLARATION

I, Godlisten Materu, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

Godlisten Materu **Date**

(MSc. One Health Molecular Biology candidate)

The above declaration is hereby confirmed;

_____ _____

Prof. Gerald Misinzo **Date**

(Supervisor)

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My genuine appreciation goes to my supervisor Prof. Gerald Misinzo, for his constructive comments, guidance and encouragement. Without his contribution, this work would not have been in its present complete form. I certify his role and I will cherish, honour and keep in memory his kindness and mentorship.

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DEDICATION

This work is dedicated to all those people who have made this course a success both directly and indirectly. Special thanks to my family and friends for standing with me throughout the entire course as a sign of appreciation I would like to say, may our Almighty God bless you.

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LIST OF ABBREVIATIONS AND ACRONYMS

ADLA	Acute (bacteria) Dermatolymphangio-Adenitis
AT	adenine-thymine base ratio
BLAST	Basic Local Alignment Search Tool
bp	base pair
CDC	Centres for Diseases Control and Prevention
CIs	confidence intervals
DDT	dichlorodiphenyltrichloroethane
DEC	diethylcarbamazine citrate
DNA	deoxyribonucleic acid
GC	guanine-cytosine base ratio
GPELF	Global Programme for Elimination of Lymphatic Filariasis
Ig E	immunoglobulin E
L1	stage larvae one
L2	stage larvae two
L3	stage larvae three
LFEP	Lymphatic Filariasis Elimination Programme
MDA	mass drug administration
Min	minute
MLE	maximum likelihood estimates
ml	millilitre
MRCC	Medical Research Coordination Committee
NCBI	National Center for Biotechnology Information
NIMR	National Institute for Medical Research
NLFEP	National Lymphatic Filariasis Elimination Programme

ºC	degree Celsius
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
TAE	Tris Acetic EDTA buffer
tRNA	transfer ribonucleic acid
WHO	World Health Organization of the United Nations
x *g*	centrifuge rotor speed
µl	microlitre

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Lymphatic filariasis, resulting from infection with the mosquito-borne filarial nematode *Wuchereria bancrofti*, is a disfiguring and disabling disease (Simonsen *et al*., 2009). It is a cause of severe suffering and a socio-economic burden in endemic communities. The main vectors are *Anopheles gambiae* and *Anopheles funestus*. However, the relative role of *Culex quinquefasciatus* as a vector is becoming increasingly important in coastal East Africa, especially in urban and semi-urban areas (Meyrowitsch *et al*., 2011).

Lymphatic filariasis is widespread and a major public health problem in many developing countries with warm and humid climate and it is one of the most prevalent neglected tropical diseases. Current estimates suggest that more than one billion people live in endemic areas and are at risk of infection, and more than one third of these are in Sub-Saharan Africa (Simonsen *et al*., 2008). In Tanzania, an estimated 34 million people at risk, while six million people are already affected by lymphatic filariasis. Lymphatic filariasis is widespread in Tanzania; particularly high endemicity is seen along the coast of the Indian Ocean and in areas adjacent to the great lakes (Malecela *et al*., 2008).

The parasites are transmitted to humans when infected mosquito vectors deposit infective larvae onto the human skin (Simonsen *et al*., 2009). The larvae penetrate the skin, migrate to the lymphatic vessels, and develop into male and female adult worms over a period of months. Mature and fertilized female worms release large numbers of minute microfilaria, which circulate in blood. Microfilaria ingested by a vector during a blood meal will develop to infective larvae in about 10-14 days. These migrate to the mosquito's proboscis

and may then be transmitted to a new human host during a subsequent blood meal. The mosquito vectors thus play an essential role in maintaining the life cycle and disseminating the infection (Bartholomay, 2002).

Clinical disease primarily results from damage caused by the adult worms in the lymphatic vessels. The common clinical manifestations (e.g. acute filarial fever, lymphoedema, elephantiasis, hydrocele) can cause considerable incapacity to the affected individuals, with consequent loss of income and social and psychological stress, and lymphatic filariasis has been recognized a leading cause of long-term disability in the world (Zeldenryk *et al*., 2011).

The principal measure currently recommended for lymphatic filariasis control is annual community-wide mass drug administration (MDA) of two drug combinations to identified communities in endemic areas (Ottesen, 2006). Tanzania was one of the first countries in Africa to initiate implementation of control, with the Tanzanian National Lymphatic Filariasis Elimination Programme (NLFEP) being launched in 2000 (Malecela *et al*., 2009).

1.2 Problem Statement and Justification

It has previously been shown in Tanzania that MDA treatment regimen drastically reduce the *W. bancrofti* microfilarial load (Simonsen *et al*., 2004). Other studies reveal the decrease in transmission lymphatic filriasis coupled with decline in anophelene mosquitoes (Derua *et al*., 2012). Although a decline in anopheline mosquitoes have been documented in Tanga, information on vector burden, vector infection rate with *W. bancrofti* and different strains of *W. bancrofti* circulating in the vectors is still lacking. Thus, this study investigated different strains of *W. bancrofti* in mosquitoes responsible for

lymphatic filariasis transmission and whether this is linked to epidemiology and/or transmission of the disease. It moreover assesed vector burden and vector infection rate with *W. bancrofti.*

The findings from this study will provide a comprehensive resource to the scientific community that will help to monitor and assess the changes taking place in the vector populations, and to elucidate the consequences for the transmission and control of the infection. This will underpin the development of new and urgently needed interventions such as vaccine development.

1.3 Objectives

1.3.1 Main objective

The overall objective of this study was to investigate strains of *W. bancrofti* in mosquitoes responsible for lymphatic filariasis transmission of Pangani District, north-eastern Tanzania.

1.3.2 Specific objectives

i. To determine the abundance of lymphatic filariasis vectors in Pangani District,

ii. To determine *W. bancrofti* infection rates in mosquitoes, and

iii. To characterize *W. bancrofti* in mosquitoes responsible for lymphatic filariasis transmission in Pangani District.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Lymphatic Filariasis

2.1.1 Aetiology

Lymphatic filariasis, also known as elephantiasis, is one of the major parasitic diseases in the tropics. Human infection with the parasites results in damaging the lymphatic vessels and causing a large range of temporary and permanent disabilities. Lymphatic filariasis is particularly associated with disfigurement of grossly swollen limbs and genitals. It is now regarded as one of the few economically important, infectious diseases of the world (Molyneux and Zagaria, 2002). The disease results from infection with the mosquito-borne parasitic nematode *W. bancrofti* (Rwegoshora *et al.,* 2007). In urban areas, *C. quinquefasciatus* has been shown to be the main vector of the parasite (McMahon *et al.,* 1981).

2.1.2 Transmission

Ingested microfilaria by the mosquito, travel from the mouthpart to the foregut and midgut of the mosquito. Within hours, the microfilaria passes through the single cell layer of the midgut epithelium to enter the haemolymph. In the midgut microfilaria can exsheath (Chen and Shih, 1988), or during the migration across the midgut, the sheath is damaged, facilitating exsheathment in the haemocoel. In the thoracic muscles, the microfilaria differentiates into the first stage larvae (L1). They then develop into the characteristic sausage-shaped second stage larvae (L2) (Smyth, 1996). It is believed that interrupting the development at this stage can result in the break of transmission (Omar and Zielke, 1978). The parasite subsequently develops into the third stage larvae (L3), also called the infective stage. At this L3 stage, there is a 4 to 6 times increase in size and the whole

development process may last between 10-12 days, under optimal conditions, mainly temperature (Lardeux and Cheffort, 1997). The infective larvae migrate into the head tissues and proboscis of the mosquito to be transmitted subsequently during blood feeding. During feeding, the infective larvae escape via the proboscis from haemolymph and enter the puncture wound made by the mosquito, hair follicles, or other abrasions. Thus, the transmission of filarial worms is highly inefficient (Bartholomay, 2002) and requires many successful bites from infective mosquitoes.

In humans, the infective larvae migrate to the nearest lymph gland where they mature into the thread like adult worms in about 3 months. Development to a sexually mature worm requires about nine months in the host (Smyth, 1996) while the average incubation time before patency is about 15 months. The mature adults can live in the human host for 5 to 10 years. After mating, the viviparous female produces microfilaria which move through the circulatory system and collect in arterioles of the lungs during the day and emerge into the peripheral blood at night, when night biting mosquitoes are most active (Nutman, 1995).

2.1.3 Pathogenesis

In filarial endemic areas, there are three groups of patients recognized (Melrose, 2002). The first group, considered endemic normals, are exposed to the nematode but have not been infected (Melrose, 2002). The second group have been exposed, infected, and have microfilaria in their peripheral circulatory system, but remain asymptomatic (Melrose, 2002). Asymptomatic infections can go undetected for years, and with lymphatic filariasis it may eventually result in internal damage which is not easily diagnosed. The third groups are those who are chronically infected and present with lymphoedema (which affects 16 million people), hydrocoele and elephantiasis (Melrose, 2002; Shenoy, 2008).

Acute (bacterial) dermato-lymphangio-adenitis (ADLA), another condition that can result from infection, presents with fever, chills, swelling and lymphoedema ADLA usually occurs when an adult worm dies and the lymph vessels surrounding it are inflamed due to the host's immunological response (Pfarr *et al*., 2009). ADLA normally occurs in older children and youth and remains with the infected individual throughout life. Chronic ADLA attacks can cause renal disease, haematuria, proteinuria, chyluria, nephritic syndrome and glomerula nephritis (Pfarr *et al*., 2009).

Patients with lymphatic filariasis can also have rheumatic problems, cystitis with urethral obstruction, fibrosing mediastinitis, tropical vaginal hydroceles and bladder pseudo tumors (Melrose, 2002). Another indication of lymphatic filariasis is pulmonary eosinophila that is characterized by paroxysmal cough and wheezing and, even though the patient harbors adult worms, there are no microfilarias in the blood. The most disabling of health problems caused by lymphatic filariasis is elephantiasis, a permanent swelling of a limb (usually lower limbs although it can effect arms, breasts and genitalia). *Streptococci* bacteria can infect the affected limb, worsening the condition (Melrose, 2002; Shenoy, 2008). Certain markers predispose patients to chronic filarial disease, including a high dose of the infectious agent, a pre-existing bacterial infection, or a specific host response (Dreye *et al*., 2000).

2.1.4 Epidemiology

Lymphatic filariasis is a widespread and a major public health problem in many developing countries with warm and humid climate and it is one of the most prevalent neglected tropical diseases (Simonsen *et al.,* 2008). It is estimated that a total of 3287 million people live in countries where the disease is endemic, and that 751 (22.85%)

million live in areas where transmission is known to occur. Of these, 72.8 million are infected with *W. bancrofti* (WHO, 1992).

Recent estimates suggest that around 120 million people living in tropical and sub-tropical environments are affected, with approximately 40 million displaying clinical signs of infection and a further 80 million people experiencing sub-clinical signs of infection (Taylor *et al*., 2010). It has also been shown that in the same area, endemicity levels are closely associated with transmission rates, which are further correlated with densities of vector populations that on the other hand, depend on availability of suitable breeding habitats in the communities (Rwegoshora *et al.,* 2007).

2.1.5 Pathology and clinical symptoms

Wuchereria bancrofti infection is usually asymptomatic. Some people can develop lymphedema, swelling, which is prevalent in the legs, but sometimes also in the arms, genitalia and breasts. The swelling and decreased flow of the lymph fluid will expose the body to skin and lymph system infections. Over time, the disease causes thickening and hardening of the skin, a condition called elephantiasis, which can be fatal. Filarial infection might also cause pulmonary tropical eosinophilia syndrome, which is mostly found in patients living in Asia. Pulmonary tropical eosinophilia syndrome can cause cough, shortness of breath, and wheezing. In addition to eosinophilia, there might be high levels of immunoglobulin E (IgE) and antifilarial antibodies.

2.1.6 Diagnosis

A blood smear is a simple and accurate diagnostic tool, provided the blood sample is taken during the period in the day when the juveniles are in the peripheral circulation (Van Hoegaerden *et al*., 1982). Technicians analyzing the blood smear must be able to

distinguish between *W. bancrofti* and other parasites potentially present. A PCR test can be performed to detect a minute fraction, as little as one pg of filarial DNA (Zhong *et al*., 1996). Some infected people do not have microfilaria in their blood. As a result, tests aimed to detect antigens from adult worms are used. Ultrasonography can also be used to detect the movements and noises caused by the movement of adult worms (Amaral *et al*., 1994).

2.1.7 Control

2.1.7.1 Chemotherapy

Strategies to eliminate lymphatic filariasis currently rely heavily on the mass distribution of drugs, free of charge, to all adults and children aged five or more living in endemic areas. The drugs being distributed are albendazole, in combination with either diethylcarbamazine citrate (DEC) or ivermectin; and recent reports suggest that more than 570 million individuals, residing in 51 countries where lymphatic filariasis is endemic, have received these drugs (Taylor *et al*., 2010).

2.1.7.2 Vector control

Before the GPELF began and before mass drug administration was used for interruption of the transmission of lymphatic filariasis, vector control appeared to be effective in some situations (Van den Berg *et al*., 2013). In areas where malaria and lymphatic filariasis are transmitted by the same species of *Anopheles* vector, interventions for malaria, such as distribution of insecticide-treated bed nets and indoor residual spraying, had a significant impact, which may have been even greater against lymphatic filariasis than malaria. For example, after several years of indoor residual spraying with DDT for malaria control, lymphatic filariasis was apparently eradicated in the Solomon Islands (Webber, 1979).

Further, use of untreated bed nets reduced vector infection rates and microfilaria prevalence in Papua New Guinea (Bockarie, *et al*., 2002).

Insecticide-treated nets are now widely used against malaria; however, for lymphatic filariasis, they are usually combined with mass drug administration (Pedersen and Mukoko, 2002). Therefore, even if the vector infective biting rate and transmission potential are frequently reduced in such situations, the independent effect of vector control cannot be measured directly. In a recent trial in Nigeria, in an area where mass drug administration could not be used because of the presence of loiasis, full coverage of all sleeping spaces with long-lasting insecticidal nets alone halted the transmission of lymphatic filariasis (Richards *et al*., 2013).

2.1.8 Geographical distribution

Lymphatic filariasis is confined to the tropics because its transmission is limited by the climatic and environmental factors that affect the distribution of its vectors. It is globally known to affect about 120 million people in at least 80 countries, and it is estimated that 1.2 billion people are at risk of infection (WHO, 2000). Of the infected individuals, a third live in India, one-third live in Africa and the rest is distributed among Asia, the Pacific and Latin America (WHO, 2000).

In sub-Saharan Africa, it is estimated that about 512 million people are at risk of infection, with almost 40 million men at risk of developing hydrocoele (WHO, 2002), whilst about 28 million are already infected. Of this number, there are 4.6 million cases of lymphoedema and over 10 million cases of hydrocoele. These represent about 20% of the global burden of the disease (Michael *et al*., 1996).

In Tanzania high endemicity is seen along the coast of the Indian Ocean and in areas adjacent to the great lakes (Malecela *et al*., 2008).

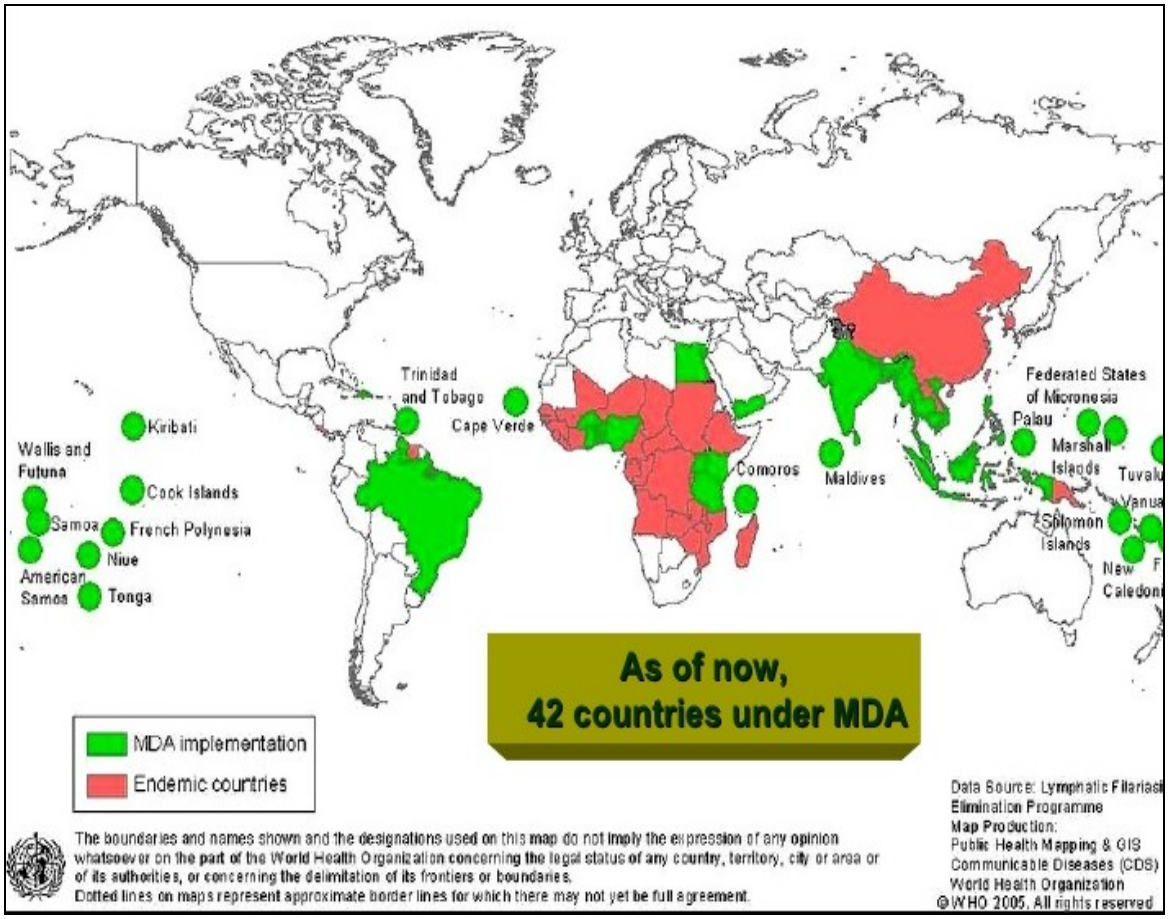

Figure 1: Global lymphatic filariasis. Endemic countries are shown by pink colour while implementation of annual mass drug administration is indicated in green colour. Source: Lymphatic Filariasis Elimination Programme (LFEP).

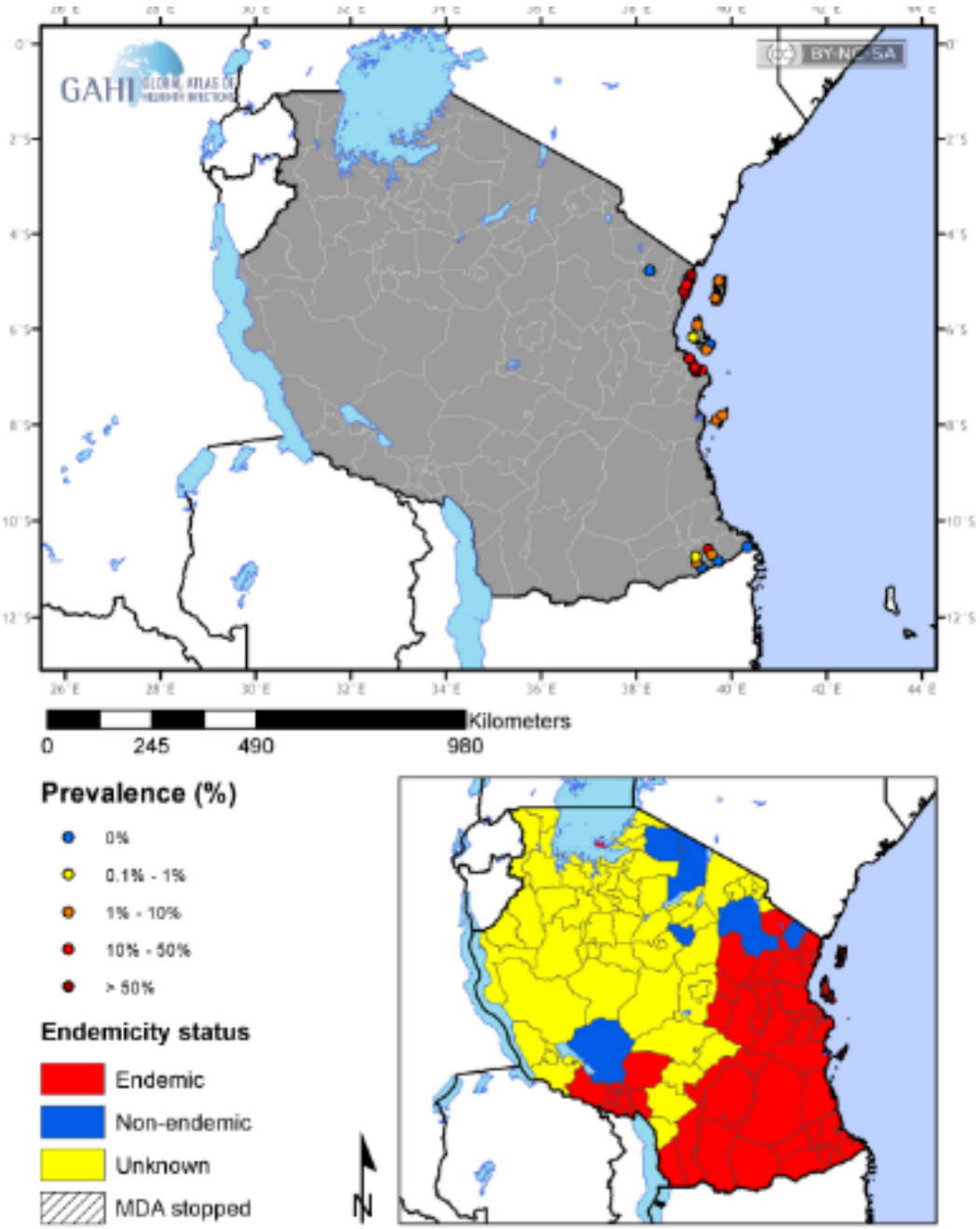

Figure 2: Distribution of lymphatic filariasis in Tanzania. The Prevalence of lymphatic filariasis survey data, post control (top map) and current endemicity status (bottom map).

2.2 *Wuchereria Bancrofti*

2.2.1 Structure of *W. bancrofti*

Wuchereria bancrofti is a filarial nematode that, as an adult, is a thread-like worm (Manguin *et al*., 2010). The female nematodes are 10 cm long and 0.2 mm wide, while the males are only about 4 cm long (Pfarr *et al*., 2009). The adults reside and mate in the lymphatic system where they can produce up to 50 000 microfilaria per day (Manguin *et al*., 2010). The microfilarias are 250-300 μm long, 8 μm wide and circulate in the peripheral blood. They can live in the host as microfilaria for up to 12 months. Adult worms take 6 to 12 months to develop from the larval stage and can live between 4 and 6 years (Bockarie *et al*., 2009).

Figure 3: *Wuchereria bancrofti* microfilaria worm. Body forms smooth (graceful) curves, has rounded anterior end and tapering tail. Both ends are free of nuclei. It has nocturnal periodicity.

2.2.2 Genomic structure of *W. bancrofti*

The length of the *W. bancrofti* mt genome is approximately 13 637 nucleotides, contains two ribosomal RNAs (rrns), 22 transfer RNAs (trns), 12 protein-coding genes, and is characterized by a 74.6% AT content (Ramesh *et al.,* 2012). The *W. bancrofti* mt gene order is identical to that reported for *Onchocerca volvulus*, *Dirofilaria immitis*, *Setaria digitata* and *Brugia. malayi*.

Wuchereria bancrofti size length of complete genome sequence, calculated by adding lengths of all scaffolds together 81.51Mbp, 29.70% GC content of scaffolds, 19 327 genes number of predicted protein-coding genes in genome, 112 tRNAs number of predicted tRNA genes in genome and eight rRNAs number of predicted rRNA genes in genome (Broad Institute, 2010).

2.2.3 Life cycle of *W. bancrofti*

The adult worms are located in the lymphatic system of the human host, where they live for 5-10 years (Vanamail *et al*., 1996; Subramanian *et al*., 2004). During their lifespan, after mating, female worms produce millions of immature microfilaria into the blood. Mosquitoes taking a blood meal may pick some of these microfilaria. Inside a mosquito, microfilaria develops in about 12 days into L3 stage larvae (L3). These L3 are infectious to human: they can enter the human body when a mosquito takes a blood meal. Some will migrate to the lymphatic system and develop into mature worms. Maturation takes 6-12 months (WHO, 1992). Microfilaria cannot develop into adult worms without passing through the developmental stages in the mosquito. The life span of microfilaria in the human body is estimated at 6-24 months (Plaisier *et al*., 1999).

The life cycle of *W. bancrofti* is shown in Fig. 4. During a blood meal, an infected mosquito introduces third-stage filarial larvae onto the skin of the human host, where they penetrate into the bite wound (1). They develop in adults that commonly reside in the lymphatics (2). The microfilaria migrates into lymph and blood channels moving actively through lymph and blood (3). A mosquito ingests the microfilaria during a blood meal (4). After ingestion, the microfilaria lose their sheaths and 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 (5). There the microfilaria develops into first-stage

larvae (6) and subsequently into third-stage infective larvae (7). The third-stage infective larvae migrate through the hemocoel to the mosquito's prosbocis (9) and can infect another human when the mosquito takes a blood meal (1).

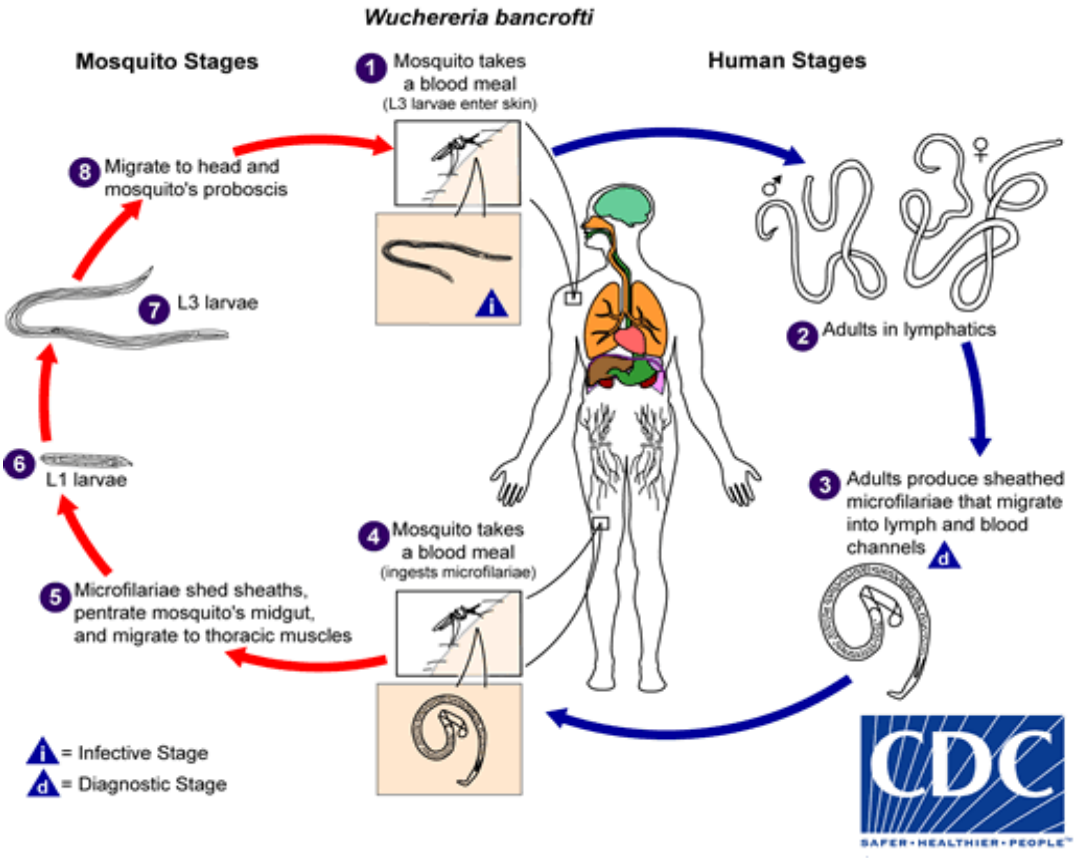

Figure 4: Life cycle for *W. bancrofti*: Source: Centres for Disease Control and Prevention.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of the Study Area

The present study was carried out in five villages of Pangani District, a district with 1830 km^2 making it the smallest district in Tanga Region. It is located in the southern part of Tanga region extending from 5^o 15.5 to 6^0 South of the Equator and from 38^o 35 to 39^o 00 East of Greenwich Meridian. It is bordered with Handeni District in the west, Indian Ocean in the East, Pwani Region to the South and Muheza District to the North. Altitude ranges from 0 to 186 meters above sea level. Mosquitoes were collected in five villages of Pangani District including Madanga, Msaraza, Bweni, Pangani West and Meka (Fig. 5).

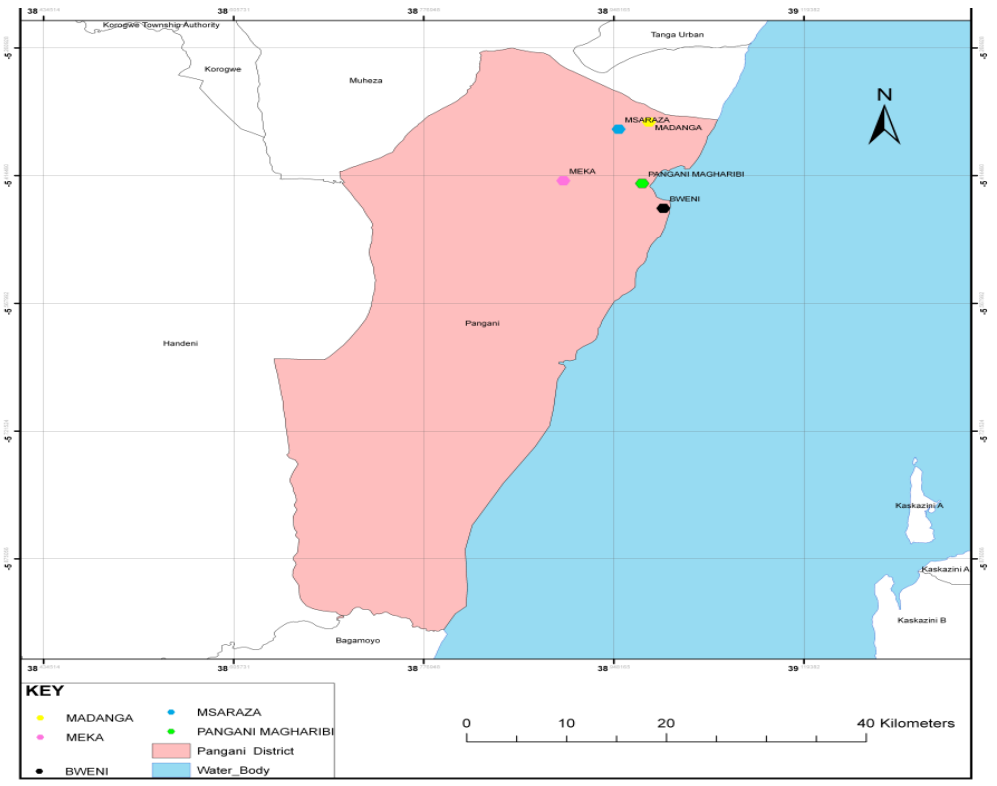

Figure 5: Map of Pangani District showing villages where mosquitoes for *W. bancrofti* detection were collected.

3.1.1 Study design

The current study employed a cross sectional design which involved trapping of mosquitoes for laboratory examination of *W. bancrofti*. The mosquito trapping was done using standard Centre for Diseases Control light traps (CDC light traps) with an incandescent light bulb (Model 512; John W. Hock Company, Gainesville, FL). The traps were hung beside beds occupied by at least one person sleeping in un-impregnated bed nets as previously described by Mboera *et al*. (1998). Briefly, the shield of the trap was left to touch the side of the net with 150cm above the floor. The light trap were set between 2000 hours and 0600 hours and retrieved in the morning at 0600 hours.

3.1.2 Sample size and sampling strategy

A total of five sentinel sites (in this case five villages) including Madanga, Msaraza, Bweni, Pangani West and Meka were randomly selected within the study area. From each village, five traditional style houses (mud walls with thatched roofs) were purposively selected for mosquito collection. Sampling was done in five sentinel sites on the Month of January 2015.

3.1.3 Mosquito storage and identification

The mosquitoes collected at each village were held separately and transported to NIMR Tanga centre, for identification using morphological features based on morphological identification keys (Edwards, 1941; Gillies and Coetzee, 1987). Female mosquitoes were pooled into pools of 20, stored in cryogenic vials with silica gels, and transported to Sokoine University of Agriculture for screening of *W. bancrofti*.

3.2 Laboratory Analyses

3.2.1 DNA extraction from mosquitoes

Deoxyribonucleic acid from pools of mosquitoes was extracted using a modification of the Qiagen DNeasy kit protocol (Qiagen, Hilden, Germany) according to the manufacturer's

instructions. Briefly, mosquitoes were crashed in phosphate buffered saline (PBS), lysed and proteins precipitated using ethanol. The supernatant was passed through a silica column followed by washing of bound DNA. Afterwards, silica was dried and DNA eluted into RNase free eppendorf tubes. DNA was stored at -20 °C until PCR were done.

3.2.2 Detection of *W. bancrofti* using PCR

Polymerase chain reaction assays for detection of *W. bancrofti* were performed using the NV1 and NV2 primers as previously described by Chanteau *et al*., 1994 and WHO 2013. The target sequence for these primers is the *Ssp I* repeat, a gene present at ~500 copies per haploid genome. Amplification with these primers yields a 188bp fragment. Each 20-µL PCR reaction contained 1× Qiagen *Taq* buffer, 50Mm MgCl2, 50 mM each of dATP, dCTP, dGTP, and dTTP, 10 pmol/ µl of NV1 and NV2 primer, 1.25 U HotStar *Taq* DNA polymerase, and 2 µl genomic DNA. PCR reactions were run on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Jurong, Singapore), and reaction conditions consisted of a single step of 95 °C for 10 minutes, followed by 94 °C for 30seconds, 54 °C for 45 seconds and 72 °C for 45 seconds. The final step was a 10-minute extension at 72 °C. PCR products were size fractionated on 1.5% agarose gels stained with GelRed (Biotium, Hayward, CA). Agarose gels were run at 100 V for 40 minutes and visualized under UV light using a gel documentation system (EZ Gel Imager, BioRed, CA). A positive control mosquito pool, known to be infected with *W. bancrofti* a kind donation from NIMR Amani Tanga Centre was included in the present study. Positive and negative controls were run concurrently with the samples to ensure that the PCR amplification is not contaminated, which can result in false positives, as well as to ensure that all the reagents are working properly.

3.2.3 Determination of infection rate of *W. bancrofti* in mosquito vectors

The computation of vector infection rates from pool screening is addressed by an application of the binomial distribution. Katholi *et al*. (1995), describe mathematical equations for such calculation. The algorithm was used to estimate the maximum likelihood of *W. bancrofti* infection at 95% confidence level in mosquitoes. In this study PoolScreen2 software obtained from the Department of Biostatistics and Division of Geographic Medicine, University of Alabama at Birmingham, USA was used. The programme relies on the fact that the PCR assay is sensitive enough to detect a single infected insect in a pool containing large number of uninfected ones.

3.2.4 DNA purification and sequencing

After identifying positive PCR product samples (i.e samples with the band of interest) from gel electrophoresis, the PCR products were purified with QIAquick PCR purification kit (Qiagen, Valencia, CA). After purification, gel electrophoresis was performed to check if the PCR products were present after purification.

Sequencing-PCR was performed in a Thermo Cycler using the purified amplicons, a single sequencing primer and a sequencing master mix The BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA). The sequencing protocol included one cycle of one minute at 95 °C followed with 50 cycles of 10 seconds of denaturation at 96 ° C, 5 seconds of annealing at 50 °C and 4 minutes of elongation at 60 °C and then a holding cycle at 4 °C. Then the Sequencing products were purified using Centri-Sep^{TM} spin columns (Princeton Separations, Inc New Jersey, USA). Followed by separation on a capillary ABI Prism 3 500 Genetic Analyzer (Applied Biosystems, Foster city, CA). The machine generates nucleotide sequence chromatograms that were obtained

as ABI files format which were assembled and edited using sequence Scanner V1.0 (Applied Biosystems, Foster city, CA). Generated nucleotide sequences were edited by BioEdit® software and used in construction of a phylogenetic tree with the aid of MEGA 6.0® using the neighbour-joining method employing the Kimura-2 parameter (Tamura *et al*., 2013). Phylogenetic analysis of obtained *W. bancrofti Ssp 1* repeat sequences was performed using the Kimura-2 parameter as implemented in MEGA 6.0. Phylogen was inferred following 1000 bootstrap replication.

3.2.5 Data management and analysis

Mosquito collection information, PCR results and infection status were recorded in special forms and thereafter entered into Excel database. Analyses of mosquito abundance were performed on the full collection data set (n= 951) and are arranged according to mosquito species and collection site. Mosquito infection rate by PCR were calculated using poolscreen2 software (Katholi *et al*., 1995), which provided maximum likelihood estimates (MLE) with 95% confidence intervals (CIs) based on the likelihood ratio.

3.2.6 Ethical consideration

This study was approved to be conducted by National ethical review committee of Medical Research Coordination Committee (MRCC) based at National Institute for Medical Research (NIMR). The ethical clearance certificate is attached in appendix 3. Permission to conduct study was sought from the authorities in the study region and the respective district and villages leaders. Moreover, written informed consent was sought from the head of household where mosquito collections were done. Other relevant authorities were informed of the study in order to get their support.

CHAPTER FOUR

4.0 RESULTS

4.1 Lymphatic Filariasis Vector Abundance

A total of 951 female mosquitoes were collected, 174 from Bweni, 301 from Madanga, 180 from Meka, 137 from Msaraza and 159 from Pangani West. Among the 951 collected mosquitoes, by far the majority were *Culex quinquefasciatus* (99.36%) followed by *Anopheles gambiae* (0.32%) and other Culicine species (0.32%) (Table 1), Appendix 5.

Table 1: Proportion of mosquito species collected for detection of *Wuchereria bancrofti* in selected villages of Pangani District, north-eastern Tanzania.

Village	*Culex quinquefasciatus*	*Anopheles gambiae* sl	Other *Culex* species
Bweni	174	0	0
Madanga	300	0	1
Meka	180	0	0
Msaraza	137	0	0
Pangani west	154	3	2
Total (%)	**945 (99.36%)**	**3 (0.32%)**	**3 (0.32%)**

4.2 Presence of *W. bancrofti* in Mosquitoes

All collected mosquitoes in Pangani District were screened for *W. bancrofti* infection. Mosquitoes were pooled in a pool of twenty mosquitoes each, resulting into a total of 47 pools. Out of 47 mosquitoes pools screened for *W. bancrofti*, 24 (51%) pools tested positive and 23 (48%) tested negative.

Positive pools produced a PCR product of approximately 188 bp, an expected size after *Ssp I* amplification using NV1 and NV2 primers (Chanteau *et al*., 1994). An example of

the agarose gel after performing PCR for the detection of *W. bancrofti* in mosquito pools is shown in Fig. 6.

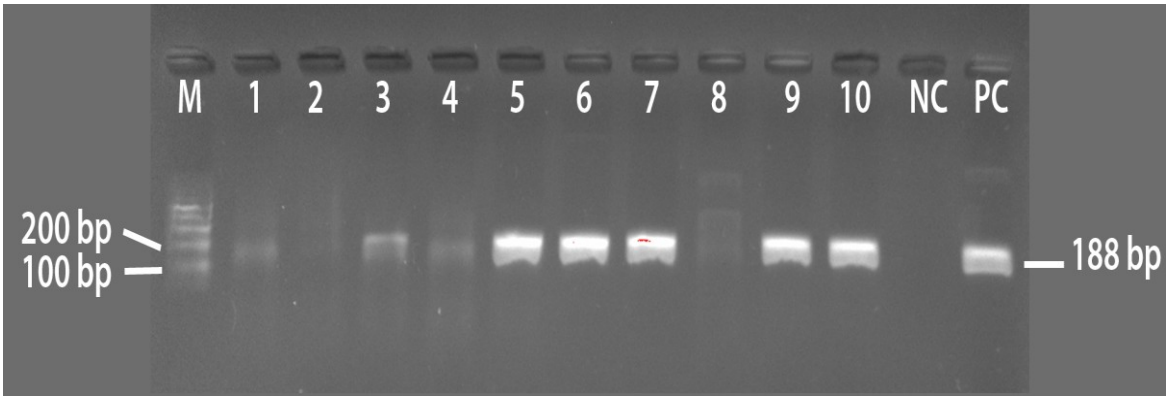

Figure 6: *Wuchereria bancrofti* detection in mosquito pools using polymerase chain reaction (PCR). PCR was used in the detection of *W. bancrofti* in mosquito pools using primers NV1 and NV2. A positive control (PC) mosquito pool known to be infected with *W. bancrofti* was used. After PCR, an expected 188 bp PCR fragment was produced in the PC and positive mosquito pools (lanes 5, 6, 7, 9 and 10). Weakly positive pools are seen in lane 1 and 3; negative pools are seen in lane 2, 4 and 8. M is 100 base pair ladder and NC is negative control.

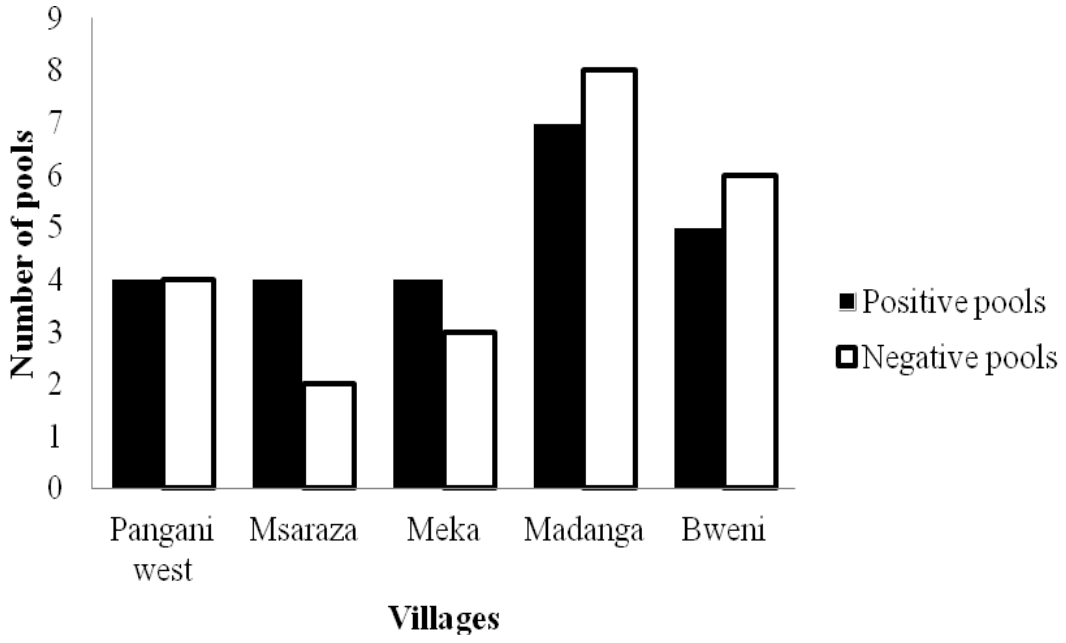

Figure 7: Proportion of *Wuchereria bancrofti* positive and negative mosquito pools collected in Pangani district. The number of positive and negative mosquito pools after performing polymerase chain reaction (PCR) for the detection of *W. bancrofti* in the different villages where mosquitoes were collected is shown.

4.3 Infection Rate of *W. bancrofti* in Mosquito Vectors

A total of 951 female mosquitoes were screened for infection with *W. bancrofti* using Poolscreen2 software (Department of Biostatistics and Division of Geographic Medicine, University of Alabama, Birmingham, USA). Poolscreen2 software (Katholi *et al*., 1995) uses maximum likelihood to estimate with 95% confidence intervals based on likelihood rates determined the infection rates. The results indicate that, Msaraza village had highest infection rate of 53.4% and Bweni village had lowest infection rate of 29.8% (Table 2).

Table 2: Infection rates of mosquitoes with *Wuchereria bancrofti* as determined by polymerase chain reaction pool screening

Village	Number of mosquitoes	Screened pools	Positive pools	Infection rate *(95% CI)
Bweni	174	11	5	29.8 (8.9-70.4)
Madanga	301	15	7	30.9 (1.15-65.4)
Meka	180	7	4	41.5 (1.04-10.8)
Msaraza	137	6	4	53.4 (1.3-14.2)
Pangani West	159	8	4	34.1 (8.6-87.9)
				Overall infection rate
Total	**951**	**47**	**24**	**35.1 (2.13-53.7)**

4.4 Molecular Characterization of *W. bancrofti* based on *Ssp I* DNA Repeat

The PCR products after amplification of *Ssp 1* DNA repeat region were sequenced (Appendix 4). Basic Local Alignment Search Tool (BLASTn) tool was used to search at GenBank nucleotide database for homologous gene sequences with that obtained from this study. Only one sequence was available at Genebank to compare with (Zhong *et al*., 1996). The absence of other *W. bancrofti Ssp 1* repeat sequences at GenBank could be due to NCBI rejection criteria for nucleotide sequences less than 200 bp long.

The nucleotide identity of the different *Ssp 1* sequences obtained from the present study with the *Ssp 1* repeat of *W. bancrofti* reported by Zhong *et al*. (1996) ranged between 80-87%. Phylogenetic analysis of the sequences obtained in the present study revealed that *W. bancrofti* obtained in the study did not belong to the same cluster (Fig. 8). In addition, the *W. bancrofti* sequences in Pangani District were not 100% identical but were closely related. *Wuchereria bancrofti* strain in some village were not closely related, example Msaraza (Fig. 8).

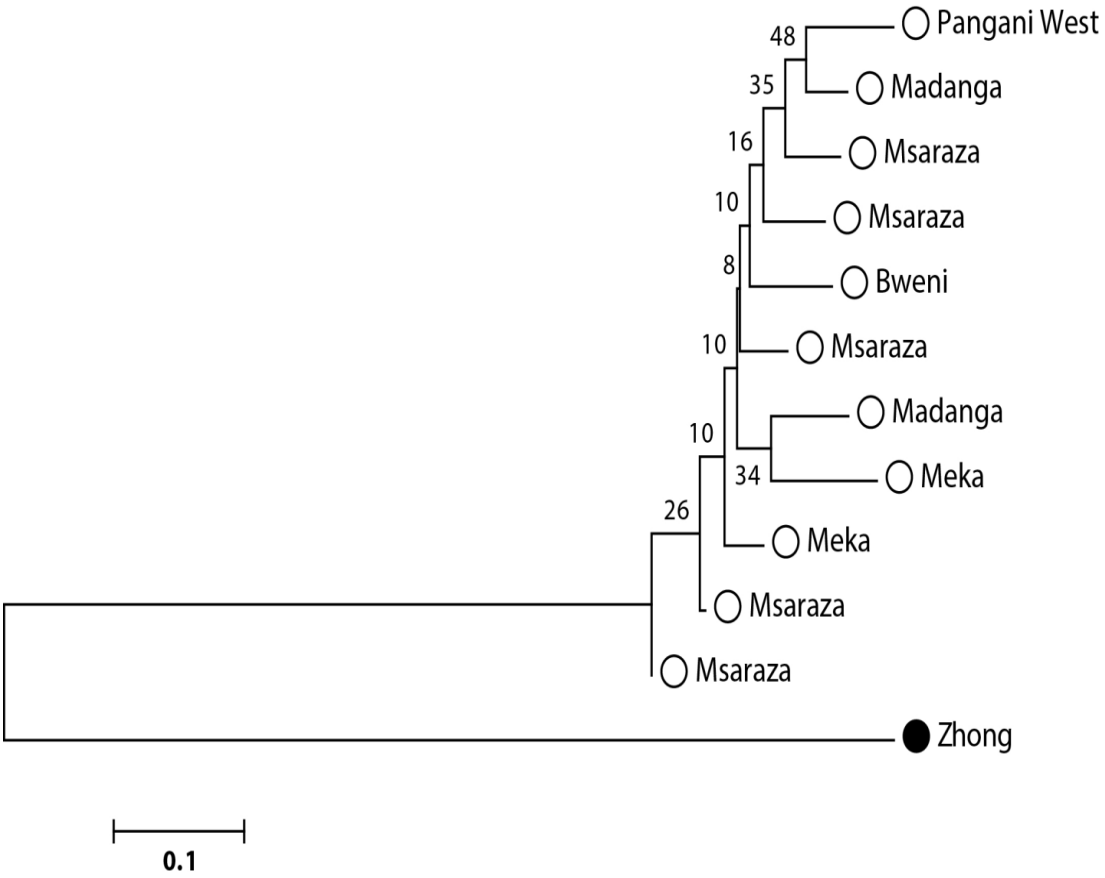

Figure 8: Neighbor-joining tree depicting *Ssp I* gene relationships of *Wuchereria bancrofti* from selected villages of Pangani district, north-eastern Tanzania. Phylogeny was inferred following 1000 bootstrap replications using the Kimura-2 parameter model and the node values show percentage bootstrap support. Scale bar indicates nucleotide substitutions per site. The samples obtained from this study are empty dots while *W. bancrofti* strain from China is marked with a filled dot.

CHAPTER FIVE

5.0 DISCUSSION

Monitoring infection rate in human population and vectors is an essential component of any lymphatic filariasis control programme for deciding when to stop MDA and for the certification of elimination of the disease. Monitoring transmission and/or infection in vectors is ideal since mosquitoes may offer a real time estimate of transmission as reported by Plichart *et al*. (2006) and Goodman *et al*. (2003), though the manifestation of microfilaria may be marginally quicker in humans. Very low-level of microfilaraemia may also not be easy to detect in human populations.

In the present study, 951 mosquitoes were collected using CDC light traps, in randomly selected villages of Pangani District, north-eastern Tanzania. Collected mosquitoes were identified based on morphological features using morphological identification keys. The results obtained from the present study indicate that *C. quinquefasciatus* was the most abundant vector species caught during the study. The mosquito abundance obtained from the present study was, *C. quinquefasciatus* (99.36%) followed by *anopheles gambiae* (0.32%) and other *Culicine* species (0.32%). These observations are corroborated by reports from Dar es Salaam by Mwakitalu *et al*. (2013) who showed that out of 12 096 vector mosquitoes caught using the light traps, great majority were *C. quinquefasciatus* (99.0%), followed by a few *Anopheles gambiae (*0.9%) and *Anopheles funestus* (0.1%).

The higher abundance of *C. quinquefasciatus* in the present study might be because mosquitoes were collected during the dry season (early January 2015) in which the mosquito's population was very low. The observed mosquitoes abundance has important implications in the transmission of both malaria and lymphatic filariasis. It is therefore

likely that the decline in anopheles mosquitoes abundance observed in the present study will have comparatively less impact on the transmission of lymphatic filariasis than that of malaria.

Mosquitoes collected and analyzed for detection of *W. bancrofti* and determination of vector infection rate in the selected area have provided an opportunity to report the presence of *W. bancrofti* and provide information on vector infection rate in Pangani District. *Wuchereria bancrofti,* infection in mosquitoes was found in all five villages, with an overall infection rate of 35.1%. Among the five villages, *W. bancrofti* was more prevalent in Msaraza (53.4%), followed by Meka (41.5%), Pangani west (34.1%), Madanga (30.9%) and Bweni (29.8%). It should be noted that these infection rate are based on all vector-borne stages of the parasite, since the PCR testing method used in the study cannot distinguish between the different larval stages. There is a need to determine the presence of infective stage of *W. bancrofti* in order to determine the transmission risk of lymphatic filariasis by these mosquitoes as previously reported by Laney *et al*. (2010).

The detection of infection in mosquito vectors is an indication that there may be positive individuals in the area. The higher rate of *W. bancrofti* in the vectors might be due to high prevalence of microfilaraemia in the human population. Simonsen *et al*. (2010) reported that, the overall prevalence of *W. bancrofti* microfilaria among individuals over the age of one was 24.5%, and it was 53.3% for *W. bancrofti*-specific circulating antigen.

While annual mass drug administration for the prevention of microfilaria transmission from person to mosquito remain the standard intervention for interrupting transmission, use of vector control to reduce the number of potential mosquito vectors is increasingly recognized as a complementary strategy in some situations (Van den Berg *et al*., 2013). A

combination of more than one vector control method will probably enhance the impact on vector populations and lymphatic filariasis transmission reduction, particularly if the methods address different stages of the mosquito's life cycle or have a different mode of action.

The nucleotide amplification, sequencing and phylogenetic reconstruction of the *Ssp I* DNA repeat revealed that, there are different strains of *W. bancrofti* circulating in mosquito vectors in Pangani district, north Eastern Tanzania. The findings showed that *W. bancrofti* strains in some village were not closely related, for instance *W. bancrofti* strain in Msaraza village. *W. bancrofti* in Pangani did not cluster with previously reported *W. bancrofti* of China using *Ssp 1* repeat region by Zhong *et al*. (1996). Though few studies have pointed to genetic and morphological variations in *W. bancrofti* populations, our findings agree with a previous study by Kumar *et al*. (2002) who reported the presence of two different genetic variants of the parasite, with high genetic divergence and gene flow in different geo-climatic regions in India. The study by de Souza *et al*. (2014) showed that there is considerable genetic variability within *W. bancrofti* populations in Ghana.

The presence of different circulating *W. bancrofti* strains in mosquito vectors call for undertaking more research on *W. bancrofti* strain circulating in the country for possible use in vaccine development. Understanding strain-specific genetic difference could provide insight into effectiveness of drug regimes, the optimal time–course of drug administration, the potential for drug resistance and vector characteristics.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

To our knowledge, this is the first study reporting the presence of different strains of *W. bancrofti* circulating in mosquito vectors in Pangani District, north-eastern Tanzania. High *W. bancrofti* vector infection rate of 35.1% was found in the present study, indicating highest chances for the presence of positive individuals in the area. Most mosquitoes collected were *C. quinquefasciatus*, the main vector of *W. bancrofti*. These calls for integrated mosquitoes control interventions in order to reduce the chances of *W. bancrofti* transmission to the population. Further studies are however required for an in-depth understanding the *W. bancrofti* larval stages in mosquitoes, their drug sensitivity and fecundity. The information would enhance strategy development regarding the impact of MDA, such as how long to run an MDA program and the optimal size of the human population treatment unit.

6.2 Recommendations

In order to further explore the results produced in this study, we recommend that further studies with a much larger sample size and employing different typing methods be conducted on the molecular characterization of *W. bancrofti*. This will enable the determination of profound conclusions on *W. bancrofti* strains. Study that will determine whether *W. bancrofti* stages in mosquito are infective need to be performed. Furthermore studies encompassing parasites from different geo-climatic regions will enhance our understanding of *W. bancrofti* strains as well as vector parasite infection status. In addition, a comparison study to investigate the prevalence of *W. bancrofti* in the human population and mosquito vector in the study area and other endemic area is of paramount, in order to draw a clear conclusion on infection status and circulating stains of *W. bancrofti* in Tanzania.

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APPENDICES

Appendix 1: Mosquito collection form

SOKOINE UNIVERSITY OF AGRICULTURE									
FACULTY OF VETERINARY MEDICINE									
Adult Mosquitoes Collected by CDC-Light Trap/Mosquito Magnet									
Village_____			House holder name:_____						
House ID_____				Trap number_____					
GPS Coordinates_____									
Mosquitoes Collected									
I. Mosquito sorting									
Genus					**Female**			**Males**	**Total**
II. Mosquito identification to species									
Mosquito Species			Physiological status					Males	Total
			Unfed		Fed	Semi gravid	Gravid		
Notes (Operational constraints etc)									

Appendix 2: Extraction of DNA from mosquitoes

The supplies needed per pool of mosquitoes are:

• 1 zinc-plated .177 calibre (4.5 mm) steel airgun shot and 2-ml tube for grinding

• 1 Qiagen DNeasy column/wash tube (provided in kit)

• 2 additional wash tubes (provided in kit) and 180 µl phosphate-buffered saline

• 40 µl proteinase K (some provided in kit; may need extra)

• 200 µl lysis buffer (provided in kit) and 200 µl 95–98% ethanol

• 1000 µl buffer AW1 (provided in kit; may need extra)

• 500 µl buffer AW2 (provided in kit) and 240 µl buffer AE (provided in kit)

• Parafilm, Pipettor and sterile tips (20, 200 and 1000 µl)

• Eppendorf centrifuge, Racks and DNA thermal cycler

Procedures

1. Extract DNA using a modification of the Qiagen DNeasy kit protocol (Qiagen, Hilden, Germany).
2. Sterilize by autoclaving 2-ml microcentrifuge graduated tubes with flat snap-top caps (e.g. Fisher Scientific) and 0.177 calibre 4.5-mm zinc-plated ball-bearings (gun pellets, e.g from Walmart, USA).
3. Place dried mosquitoes for grinding in sterile tubes, with one pool per tube.
4. Add 180 µl phosphate-buffered saline and one zinc-plated ball-bearing to each tube. Wrap Parafilm around the cap of each tube to prevent leakage or contamination.
5. Vortex the tubes with mosquitoes for 15 min to macerate them, e.g. on a Fisher Vortex Genie 2 mixer (Fisher Scientific, Waltham, Massachusetts, USA) with a Mo Bio Horizontal vortex adapter (Mo Bio, Carlsbad, California, USA).

6. Spin the tubes briefly before adding 200 µl of lysis buffer (buffer AL) and 20 µl of proteinase K to each sample. Vortex the samples briefly, and incubate at 70 °C for 10 min.
7. Add an additional 20 µl of proteinase K to each tube, and incubate the samples at 56 °C for 60 min. Then spin the incubated material at 13 000 g for 5 min, remove the supernatant and add it to 200 µl of 98% ethanol.
8. Apply this mixture of supernatant and ethanol to the Qiagen DNeasyspin column, and then wash the column twice with buffer AW1 and once with buffer AW2.
9. Elute DNA from the column into a labelled 1.5–2.0-ml tube (e.g. Eppendorf tube) by adding 125 µl of AE elution buffer (performed twice).

   The purified DNA is then ready for use in the PCR assay. Known positive and negative control DNA samples are recommended.

Appendix 3: NIMR ethical clearance certificate

THE UNITED REPUBLIC OF TANZANIA

National Institute for Medical Research
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NIMR/HQ/R.8a/Vol. IX/1834

Ministry of Health and Social Welfare
P.O. Box 9083
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Tel: 255 22 2120262-7
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10th October 2014

Mr Godlisten Materu
NIMR Tukuyu
P O Box 538,
TUKUYU, Mbeya

CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Molecular Characterization of *Wuchereria bancrofti* in Mosquitoes in Pangani District, North eastern Tanzania, (Materu G *et al*) has been granted ethical clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health and the National Institute for Medical Research, Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health & Social Welfare and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine. NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Sites: Pangani District

Approval is for one year: 10th October 2014 to 09th October 2015.

Name: Dr Mwelecele N Malecela

Signature
CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

Name: Dr Donan Mmbando

Signature
CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, SOCIAL WELFARE

CC: RMO
DED
DMO

Appendix 4: ***Wuchereria bancrofti Sssp 1*** **repeat aligned sequences**

Pangani West

>TTAAAAAATAAGGTTATACCAAGCAAACAAAAAAAAAAAAAAATTTCAAAA

AAAAATTTAAAAAAAAAATTTGCAAAAATTCCCTTTCTCCTTTGAAAATTTTA

AAAAAAAATTTCCTTTCCCCTTTTTTGGGGCCCCCGGGGG

Madanga

>TTAAAAAATAAGGTTATACCAAGCAAACAAAAAAAAAAAAAATTTTTAAA

AAAAAATATACCAAAAAAATTGGTAAAAATCCCCTTCCTCCTTGGAAAATTTT

AAAAAAAAATTCCTTTTCCCCCTTTTTGGGCCCCCCGGGG

Msaraza

>TTAAAAAATAAGGTTATACCAAGCAAACCAAAAAAAAAAAAAATTTTTCAAT

AAAAATTTTAAAAAAAAATTTGGCAAAAATTCCCTTTCTCCTTGGAAAATTTT

AAAAAAAATTTCCCTTTCCCCTTTTTTGGGGCCCCGGGGG

Msaraza

>TTAAAAAATAAGGTTATACCAAGCAAACAAAAAAAAAAAAATTTTTCAATA

AAAATATAAAAAAAAAAATTGGGGAAAATTCCCTTTCCCCCTGGAAAATTTTA

AAAAAAATTTCCCTTTCCCCTTTTTTGGGGCCCCGGGGG

Msaraza

>TTAAAAAATAAGGTTATACCAAGCAAACAAAAAAAAAAAAATTTTTCAAA

AAAAAAATTAAACAAAAAATTGGCAAAATTTCCCTTTCCTCTTTGAAAATTTT

AAAAAAAATTTCCTTTTCCCCTTTTTTGGGCCCCCGGGGG

Pangani West

>TTAAAAAATAAGGTTATACCAAGCAAACCAAAAAAAAAAAAATTTTTAAAA

AAAAATAAAAAAAAAAAAATTTCTAAAAATTCCCTTTCTCCTTGAAAAATTTA

AAAAAAAATTTTCTTTTCCCCTTTTTTGGGCCCCCGGGGG

Msaraza

>TTAAAAAATAAGGTTATACCAAGCAAACCAAAAAAAAAAAAATTTTTCAAT

AAAAATAAAAAAAAAAAATTTGGGAAAATCCCCTTTCCCCCTTGAAAATTTTA

AAAAAAAATTTCCTTTTCCCCTTTTTTGGCCCCCCGGGGG

Meka

>TTAAAAAATAAGGTTATACCAAGCAAACAAAAAAAAAAAAATTTTTCAATA

AAAATATTAACCAAAAATTTGGCAAAATTTCCTTTTCCCCCTGGAAAATTTTA

AAAAAAATTTCCCTTTCCCCTTTTTTGGGCCCCCGGGGG

Meka

>TTAAAAAATAAGGTTATACCAAGCAAACCAAAAAAAAAAAAATTTTCCAAA

AAAAATTTTACCCAAAAAATTGGCGAAAACCCCCTTCCCCCTTGGAAATAATT

AAAAAAACATTCCCTTTCCCCTTTTTTGGGGCCCCGGGGG

Madanga

>TTAAAAAATAAGGTTATACCAAGCAAACAAAAAAAAAAAAATTTTTAAAA

AAAAATTTAACCAAAAAATTTGGTAAAAATCCCTTTCCCCCTTGAAAATTTTA

AAAAAAAATTTCCTTTCCCCCTTTTTTGGGCCCCCCCGGG

Bweni

>TTAAAAAATAAGGTTATACCAAGCAAACAAAAAAAAAAAATTTTTTCTTTAA

AAATATAAAACAAAAAATTTGCAAAAATCCCCTTCCCCCTTTGAAAATTTAAA

AAAAAATTTTCCTTTCCCCTTTTTTGGGCCCCCGGGGG

Zhong

>CGTAAGGGAATTGTTTTTTTAATATTTTCAAGTATGAATGGAATTTTTAGCAA

TTTTTTTGTTTATATTTTTATTTGAATT--

ATTTTTTTTTTTGTTTGCTTGGTATAACCTTATTTTTTAATCTTTTTTAATTTTTT

TAGTT

Appendix 5: Raw data

Village	Latitude_S	Longitude E	*Anopheles gambiae sl*	*Culex quinquefasciatus*	Other *Anopheles* species	Other *Culex* species	Screened pools	PCR results (+/-) pools
Bweni	05 27.228	038 59.567	0	67	0	0		
Bweni	05 27.297	038 59.539	0	58	0	0		Positive 5
Bweni	05 27.258	038 59.546	0	30	0	0		Negative 6
Bweni	05 27. 110	038 59.618	0	19	0	0	11	
Pangani West	05 25.301	038 58.448	0	17	0	0		
Pangani West	05 25.329	038 58.300	0	14	0	0		
Pangani West	05 25.393	038 58.261	0	2	0	0		
Pangani West	05 25.506	038 58.414	0	5	0	0		
Pangani West	05 25.469	038 58.501	3	14	0	2		
Pangani West	05 25.462	038 58.502	0	43	0	0		Positive 4
Pangani West	05 25.517	038 58.488	0	52	0	0		Negative 4
Pangani West	05 25.506	038 58.480	0	7	0	0	8	
Meka	05 26.114	038 52.920	0	42	0	0		
Meka	05 26.226	038 52.988	0	79	0	0		
Meka	05 26.411	038 53.024	0	34	0	0		Positive 4
Meka	05 26.408	038 53.018	0	25	0	0	7	Negative 3
Madanga	05 21.007	038 58.870	0	68	0	0		
Madanga	05 21.025	038 58.815	0	60	0	0		
Madanga	05 21.025	038 58.755	0	103	0	0		
Madanga	05 20.985	038 58.794	0	29	0	0		Positive 7
Madanga	05 20.961	038 58.767	0	40	0	1	15	Negative 8
Msaraza	05 21.763	038 57.418	0	29	0	0		
Msaraza	05 21.807	038 57.287	0	26	0	0		
Msaraza	05 21.322	038 57.048	0	31	0	0		Positive 4
Msaraza	05 21.202	038 56.840	0	51	0	0	6	Negative 2