PREVALENCE OF *Wuchereria bancrofti* MICROFILARIAE AND ITS ASSOCIATION WITH HIV AMONG FILARIAL ANTIGENEMIA POSITIVE INDIVIDUALS IN KYELA DISTRICT, TANZANIA

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2024

Prevalence of *Wuchereria bancrofti* Microfilariae and its Association with HIV Among Filarial Antigenemia Positive Individuals in Kyela District, Tanzania.

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Molecular Medicine of the Jomo Kenyatta University of Agriculture and Technology

2024

DECLARATION

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

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DEDICATION

This work would not have been possible without the support of many individuals. First and foremost, I would like to take this opportunity to just give thanks to God Almighty for granting me good health throughout the journey of completing this project. It would have not been easy without the moral and spiritual support including prayers from my family, I would like to express my sincere gratitude to my dear wife Joyce, my lovely kids Joshua, Joel and Jerminah, and the family. They absolutely made me reach this far.

ACKNOWLEDGEMENT

I am extremely appreciative of the willingness of Elimination and Monitoring of the Impact of New Interventions (EMINI) study participants as well as field and laboratory staffs to take part in the study. Indeed, I would like to express my special thanks to Dr Inge Kroidl, a Principal investigator of RHINO (Risk of HIV Infection through Nematodes) study for allowing this project which is part of RHINO's objective to be part of my MSc project and for the financial support that the RHINO study provided throughout my postgraduate training. I am greatly indebted to RHINO study team for their tremendous encouragements, advices and team spirit. I would also like to thank my academic supervisors from JKUAT, KEMRI Graduate School Kenya, and UDSM-MCHAS/NIMR-MMRC Tanzania (Dr. Eric Lelo, Prof. Daniel Kariuki and Dr. Mkunde Chachage respectively) for their valuable guidance, advice, supportive and constructive feedback and all the necessary support that contributed much to the successful completion of this research project.

It wasn't easy to accomplish this work without the technical support and assistance from experienced lab scientists and technicians from both the Institute of Immunology, Microbiology, Molecular biology and Parasitology (IMMIP) Bonn Germany, and NIMR-MMRC Mbeya Tanzania, in one way or another they made me reach this far. Sincere gratitude is extended to (IMMIP) staffs, specifically Tilman Aden and Christina Lämmer at the molecular laboratory for their technical and expert support throughout the sample analysis. I personally would like to offer special thanks to my dear wife and family, words are not enough to express how grateful I am. Hopefully, their prayers were what sustained me thus far.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ACRONYMS AND ABBREVIATIONS

bp	Base Pair
CDC	Centre for Disease Control
cDNA	Complementary Deoxyribonucleic acid
CFA	Circulating Filarial Antigen
CI	Confidence Interval
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
ELISA	Enzyme-Linked Immunosorbent Assay
EMINI	Evaluation and Monitoring of the Impact of New Interventions
GAELF	Global Alliance for Eliminating Lymphatic Filariasis
gDNA	Genomic Deoxyribonucleic Acid
HIV	Human Immunodeficiency Virus
IFN	Interferon
IgG	Immunoglobulin G
IL	Interleukin
IRR	Relative Incidence Ratio
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
LF	Lymphatic Filariasis
MCHAS	Mbeya College of Health and Allied Sciences
MDA	Mass Drug Administration
MF	Microfilariae
MF-	Microfilaria Negative
MF+	Microfilaria Positive
MMRC	Mbeya Medical Research Centre
NIMR	National Institute for Medical Research
NLFEP	National Lymphatic Filariasis Elimination Programme
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid

RHINO	Risk of HIV Infection through Nematodes
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TNF	Tumor Necrosis Factor
UDSM	University of Dar es Salam

ABSTRACT

Wuchereria bancrofti is a filarial nematode causing a chronic debilitating infection known as lymphatic filariasis (LF). It accounts for 90% of all lymphatic filariasis cases worldwide. The disease is characterized by clinical manifestations of lymphoedema of the lower limbs (elephantiasis) and hydrocele that leads to severe morbidity of the infected person. The previous study described increased susceptibility to Human immunodeficiency virus (HIV) in individuals infected with *W. bancrofti*, as measured by circulating filarial antigen (CFA) a marker which released is by the adult filarial worms. During that study microfilaria test wasn't done which would have been help to discriminate between patent and latent infection and the link to HIV susceptibility. Main objective of this study was to determine prevalence of *W. bancrofti* microfilariae and its association with HIV among CFA positive individuals who were annually followed up for five years. The present study was retrospective laboratory based that used bio-banked blood samples collected from people living in Kyela district who were CFA positive but HIV negative at baseline. A nonprobability purposive sampling technique was used to select a total 396 bio-archived blood samples. DNA was extracted and *W. bancrofti* microfilaria chitinase gene was detected by using Polymerase Chain Reaction (PCR). Statistical analysis was done using Pearson test, where p value <=0.005 was considered significant. Out of 396 samples, 350 were analyzed, of which 12/350 (3.4%) were positive for *W. bancrofti* microfilaria chitinase PCR. Among them 9/350 (2.6%) were samples collected from female and 3/350 (1.7%) from males (Pearson correlation, p=0.062). During the four-year follow-up period (1085 person-years), 22 individuals became HIV infected, resulting in an overall HIV incidence of 2.0 per 100 person-years. The incidence of HIV in *W. bancrofti* MF chitinase positive subjects was significantly higher than of MF chitinase negative study subjects (8.7 vs 1.8 cases per 100 person years, p=0.014). Multivariable log link binary regression was used to determine the association, when adjusted for age, sex and economic status, the risk of HIV infection was about 5 times higher in MF infected individuals than those without MF, despite active *W. bancrofti* infection (IRR 4.58; 95% CI: 1.37 – 15.4, p=0.009). As observed that MF positivity increased the risk to HIV infection, therefore MF screening and treatment should be considered as one of the prevention and control strategy in areas endemic to HIV and filariasis.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Lymphatic filariasis (LF) is a vector-born parasitic neglected tropical disease caused by filarial nematodes. About 90% of global cases of LF are caused by *Wuchereria bancrofti (W.bancrofti),* the blood parasite which affects the lymphatic system of the human causing lymphedema (elephantiasis) of the lower limbs and hydroceles leading to severe morbidity of the affected individual which may cause permanent disability (CDC, 2021; Cromwell et al., 2020). The burden of LF has now remarkably reduced globally, from reported 199 million cases in the years 2000 to 51 million infected individuals in 2018. This great achievement was made possible by the Global Alliance for Eliminating Lymphatic Filariasis (GAELF) through mass drug administrations (MDA) (Cromwell et *al.,* 2020; Herricks *et al.,* 2017). In Tanzania, particularly in Mbeya region along the Lake Nyasa where the MDA started in 2009 through the Tanzania Lymphatic Filariasis Elimination Programme, the prevalence of LF was earlier reported to be 42.3% among adults individuals in the Kyela district through detection of circulating filarial antigen (CFA) (Kroidl *et al.,* 2016a). The prevalence of microfilaria was reported to be 5.2% among CFA positive individuals in Mkinga District North-eastern Tanzania (Fimbo et al., 2020b). After several MDA rounds in Kyela district, prevalence of LF by circulating filaria antigenemia was significantly reduced from 35.1% to 1.7% as it has recently been reported (Mnkai *et al.,* 2022).

Transmission of *W. bancrofti* filarial worms is through an infected mosquito of the genus Anopheles, Culex and Aedes. Following bite of an infected mosquito, an invasive stage 3 larvae (L3) is introduced into human host where it will develop into adult worms dwelling in the lymphatic system and producing millions of microfilariae (MF) the transmission stage of the infection which circulate in human's peripheral blood (CDC, 2021).

The adult filarial worms which dwell in the lymphatic vessels of the host can live for six to eight years and produce millions of microfilariae (MF), the transmission stage of LF

infection which circulate in human peripheral blood. During their life time, adult filaria worms release antigen which also circulate in the host's blood. These CFA can be used in laboratory diagnosis of LF. (Dolo *et al.,* 2018; Nutman *et al.,* 2015).

Bancroftian filariasis can be diagnosed through various laboratory methods such as microscopic examination of microfilariae through night blood smear, detection of specific antigen or antibody through ELISA and detection of filarial nucleic acids through polymerase chain reaction (PCR) based assays (Singh *et al.,* 2013). Microscopic detection of MF in blood smear requires expert manpower, is a labour-intensive work and needs blood to be collected during the night due to nocturnal periodicity of the parasite (Hertz *et al.,* 2020). Immunoassays developed and introduced during the 1980s for the detection of circulating filarial antigen by using monoclonal antibodies. These tests are quick, require neither expertise no sophisticated equipment and are not limited to a large blood volume . The use of molecular based assays particularly polymerase chain reaction (PCR) in the diagnosis of LF have been reported and remain the most promising in the detection of MF or adult worm DNA or RNA in human blood and other specimens (Almeida et al., 2018a; Ximenes et al., 2014).

Infection with human immunodeficiency virus (HIV) has been reported to be associated with parasitic infections such as malaria and helminths (including LF) in endemic areas (Kroidl *et al.,* 2016a; Nielsen *et al.,* 2006; Simon, 2016; Yegorov *et al.,* 2019). Comparative to MF negative *W. bancrofti*-infected individuals, MF positive people exhibit suppression of the cytokines interleukin (IL) 5, 6 10, and 17, as well as interferon (IFN), and tumor necrosis factor (TNF) (Arndts et al., 2012). In previous study, more than two fold increased risk of HIV infection in individuals infected with LF was reported, where CFA was used as a diagnosis marker in study population (Kroidl *et al.,* 2016b). However, MF was not detected in the initial previous study cohorts, and the association of MF and HIV infection has not been reported in Tanzania. Therefore, the current study was conducted to retrospectively evaluate the prevalence of *W. bancrofti* microfilariae and the association with HIV infection using biobanked blood samples collected from the same previous study population.

The archived blood samples from CFA positive and HIV negative participants from the previous study were used in current study to determine the presence or absence of MF by detecting *W. bancrofti* microfilaria specific chitinase gene using the established real-time PCR.

1.2 Problem Statement

Previously, Kroidl reported a 2.3-times increased HIV susceptibility among individuals infected with *W.bancrofti* in contrast to those filarial-uninfected participants (Kroidl *et al.,* 2016b). The incidence was determined by the detection of CFA without considering the microfilarmic or amicrofilaremic as an attribute of the increased risk to HIV in *W. bancrofti*-infected individuals. It has been demonstrated that microfilaremia is associated with weakened immune responses to filaria and other parasitic antigens (Layland, *et al.,* 2012). Considering that CFA is specific for adult filarial worms and also that the detect microfilaria by microscopic examination would not have been possible for the study of Kroidl *et al.,* because the blood was collected during the daytime contributing to absence or low level of microfilaria in the blood. To fill this gap, a developed real-time PCR method which targeting *W. bancrofti* microfilaria specific chitinase gene was used to determine the prevalence of *W. bancrofti* MF to complement the previous reported increased HIV incidence and determine the association of microfilaremia or amicrofilaremia with the risk of HIV acquisition of the same study cohorts from the archived blood samples.

1.3 Justification

Lymphatic filariasis is diagnosed using various techniques, including the detection of MF in thick blood smear. This technique not only requires expertise and labor-intensive work in morphologically identification of MF but also needs blood samples to be collected during the night when MF transmission occurs (Hertz *et al.,* 2020).The development and commercialization of immunoassays which detect filarial antigens revealed that most individuals with detectable circulating filarial antigen have adult worms that produce microfilariae (Arndts *et al.,* 2012). Several authors have reported the use of molecular methods, particularly PCR in the diagnosis of LF in human blood samples (Almeida *et al.,* 2018a; Fink *et al.,* 2011; Ximenes *et al.,* 2014). To complement the previous

evaluation that individuals infected with filariasis (CFA positive) have an increase susceptibility to HIV as compared to CFA negative (Kroidl *et al.,* 2016b), we have established and use a real-time PCR method to detect *W. bancrofti* microfilaria-chitinase gene to retrospectively asses the prevalence of *W. bancrofti* MF and the association between MF status (microfilaremia/amicrofilaremia) with HIV incidence from biobanked blood samples collected from the previous study.

1.4 Research Questions

1. What was the prevalent of *W. bancrofti* microfilariae among filarial antigenemia positive individuals in Kyela district?
2. What is correlation between HIV incidence and *W. bancrofti* microfilaria positivity among individuals with filarial antigenemia?

1.5 Null Hypothesis

Microfilaremia is no associated with increase susceptibility to HIV in people infected with *W. bancrofti* microfilaria.

1.6 Objectives

1.6.1 General Objective

To determine prevalence of *W. bancrofti* microfilariae and its association with HIV among filarial antigenemia positive individuals in Kyela district, Southwest Tanzania.

1.6.2 Specific Objectives

1. To determine the prevalence of *W. bancrofti* microfilariae among filarial antigenemia positive individuals in Kyela district.
2. To determine the correlation between *W. bancrofti* microfilariae and HIV incidence among filarial antigenemia positive individuals in Kyela district.

CHAPTER TWO

LITERATURE REVIEW

2.1 History and Biology of *Wuchereria bancrofti*

Wuchereria bancrofti is a roundworm (nematode) a leading causative agent of lymphatic filariasis among three filarial worms. *Wuchereria bancrofti* was discovered and named in honour of Brazilian physician Otto Wucherer and British pathologist Joseph Bancrofti in 1920s. The pathogen is taxonomy classified under family *Filariidae,* genus *Wuchereria* and specie *bancrofti.* (Otsuji, 2011).

Adults *Wuchereria bancrofti* worms are pseudocoelomate (coelomic cavity is not surrounded by mesoderm) cylindrical, thread-like nematode. The body is covered with a thick cuticle with syncytial (multinucleated) skin. Female are longer (28-40mm long , 100μm wide) and wider than male (80–100 mm long×240-300 μm wide) worms (Kaushal *et al.,* 2012).The gravid female worms which dwell in lymphatic vessels of human host, they produce microfilariae (244–296 μm long and 7 μm wide) the larvae which lodge in blood circulation with a marked nocturnal periodicity which means that they are oud in high numbers in the peripheral blood around 3-4h at midnight with a marked low numbers or nearly absent during the daytime (Paily *et al.,* 2009). Filarial worm *Wuchereria bancrofti* which is responsible for the filariasis/elephantiasis is transmitted by five different mosquito vectors namely *Culex quinguefasciatus, C. pipiens molestus, C. pipiens pipiens, Anopheles sinensis, A. gambiae, A. melas A. merus and A. maculates* (CDC, 2013; Jones *et al.,* 2018).

Parasite *Wuchereria bancrofti* is globally distributed in around 72 countries mostly in Sub-Saharan Africa, Asia, the Western Pacific and some areas in the Americas (Cromwell *et al.,* 2020). In Tanzania, the Bancroftian filariasis highly distributed along the coastal regions though some areas in southern highland of Tanzania specifically Kyela district which is located along Lake Nysa was reported to have Bancroftian filariasis cases (Kroidl *et al.,* 2016a). Bancroftian filariasis as other lymphatic filariasis is an asymptomatic disease until some years after infection when it may be manifested as severe immunopathology of lymphedema and/or hydrocele (Kar *et al.,* 2017).

Diagnosis of lymphatic filariasis can be confirmed through different laboratory techniques such as microscopic detection of MF, rapid tests that target either specific antibody or antigen (e.g., CFA), ELISA and PCR based assays (Singh *et al., 2013).* Sensitivity and specificity vary among the tests. PCR based assays is reported to have high sensitivity and specificity in the detection of microfilariae and adult worm in blood samples (Almeida *et al.,* 2018b).

2.2 Life Cycle and Transmission of *Wuchereria bancrofti*

Wuchereria bancrofti is transmitted to human by an infected mosquito which introduce the third stage infective larvae (L3) when it takes its blood meal. The L3 entering the host through a bite wound and migrates to the lymphatic tissue through lymphatic vessels where they develop into adult worms. They sexually reproduced and bear sheathed microfilariae which migrate into the blood circulation through lymphatic system. The circulating microfilariae are then ingested by a new mosquito when taking its blood meal from an infected human. In the mosquito, ingested microfilariae develop into first stage larvae (L1), second stage larvae (L2) and third stage larvae (L3) then the cycle completed when L3 is infected to another person (Figure 2.1). *Wuchereria bancrofti* and *Brugia malayi* microfilariae belong to nocturnal periodicity microfilariae which are found in peripheral blood of an infected person in high concentration during mid-night hours only and not at other time (WHO, 2021)

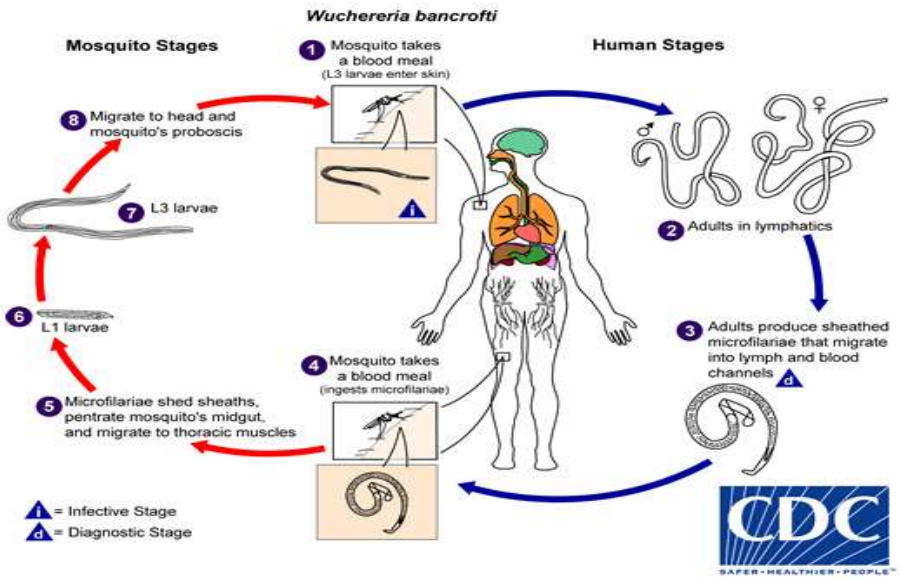


Figure 2.1: Life Cycle and Transmission of *Wuchereria bancrofti*

Source: (CDC, 2013)

2.3 Diagnosis of *Wuchereria bancrofti*

Wuchereria bancrofti and other species which cause lymphatic filariasis can be diagnosed through various laboratory tests. The diagnosis approaches which include identification of microfilariae by microscopic examination, detection of antibody and/or detection of antigen and detection of nucleic acids. The diagnosis efficacy of each approach differs based on various factors such as developmental stages of the parasite, the region of endemicity that the patient ha migrated from or visited whether the patient is categorized as endemic or non-endemic (WHO, 2021). Differences in the usefulness and sensitivity of the various diagnostic techniques currently employed are indicative of variations in the morphological and genetic constitution of the worm.

2.3.1 Microscopic Examination of *W. bancrofti*

W. bancrofti MF can easily be distinguished from *B. malayi* and *B. timori* MF by its non-densely packed nuclei, the absence of two isolated nuclei at the tip of the tail, and the

presence of nuclei in the head space. There are limited reports that detailing intra-specific variation among *W.bancrofti* MF (Kaushal *et al.,* 2012; Paily *et al.,* 2009). Historically, lymphatic filariasis has been diagnosed through the identification of circulating microfilariae in the peripheral blood of the infected person. However, the detection of MF in all species does imply the possibility of transmission, MF detection is not as sensitive as antigen tests when selecting an initial diagnostic test due to the fact that merely 50-70% of LF endemic cases exhibit MF due to various reasons such as the presence of single-sex worms, immaturity of the female worms leading to their incapability of reproduction, clearance of MF from the circulation by the immune response, or the effete state of the female worms resulting in their non-reproductive status (Njenga *et al.,* 2007). Differences in immune sensitization between endemic and non-endemic has been reposted to be one of the reasons contributed to around 0.2% microfilaremia in non-endemic LF cases (Ottesen, 1992). In contrast to other available diagnostic tests, the identification of MF is dependent upon the precise timing of the blood collection (Cox-Singh *et al.,* 2000). The temporal occurrence of the highest concentration level of circulating MF in the bloodstream exhibits variability across LF endemic regions and is believed to depend upon the biting patterns of the indigenous mosquito vector. In the region where mosquitoes exhibiting nocturnal biting behavior, the presence of MF in the circulation is typically observed during the night time hours with the peak levels occurring between 2200 and 0200 hours (Manguin *et al.,* 2010). Detection of microfilariae it has been a long-standing and established method, however it has been superseded by antigen detection tests for *W. bancrofti* but not for the other species (Weil & Ramzy, 2007).

2.3.2 Antibody Detection

Identification of circulating anti-filarial antibodies as a marker for exposure and current or previous infection have been employed using several crude and recombinant filarial antigens through the innovation of recombinant DNA technology which increased both sensitivity and specificity (Lammie *et al.,* 2004; Njenga *et al.,* 2008). Prior to the advancement in recombinant DNA technology, there existed significant serological cross-reactivity among the antigens of the three lymphatic filarial species as well as other filarial species. For instance, it has been reported that Brugia Rapid commercial assay, which uses a *B. malayi* antigen to identify anti-filarial IgG4 antibodies, exhibits comparable

sensitivity towards antibody targeting *B. timori* (Supali *et al.,* 2004) The aforementioned test may also identify antibodies against *W. bancrofti* infection, although the sensitivity has been decreased. Therefore, the current antibody assays are not helpful for the detection of variability of lymphatic filariae despite of their efficacy in diagnostic applications (Weil *et al.,* 2011)

2.3.3 Antigen Detection

In order to address the requirements of the Global Programme to Eliminate Lymphatic Filariasis (GPELF), novel diagnostic tools utilizing immunological methods and recombinant antigens have been devised (Lammie *et al.,* 2004; Rahmah *et al.,* 2001). These tools are designed to either capture antibodies from the sera of infected individuals or general antibodies against specific filarial antigens, enabling direct capture of the corresponding antigens from the sera (Ravishankaran *et al.,* 2015; Steel *et al.,* 2013). Several recombinant antigens have been commercially used in the identification and detection of filarial infection in human blood sample. The World health organization (WHO) defined the circulating filarial antigen (CFA) tests as the gold standard for the diagnosis of LF, particularly Bancroftian filariasis. The CFA test are capable of detecting the presence of adult worms antigen circulating in the host bloodstream, which serves as an indicator of the existence of living adult worms, consequently an active infection (Weil *et al.,* 1999).These novel tools offer the benefit of high sensitivity in comparison to other parasitological techniques and are amenable to samples collection at any hour of the day. Additionally, they yield prompt outcomes and necessitate minimal infrastructure (C *et al.,* 2015; Pastor *et al.,* 2021).

2.3.4 Nucleic Acid Detection

The utilization of molecular biology techniques has led to significant progress in the development of species-specific primers for the PCR-based identification of disease pathogens. Real-time PCR is a highly effective method for rapid and efficient detection of pathogen DNA, and is increasingly being utilized as a replacement for conventional PCR and microscopy in the diagnosis of various parasitic infections. It's ability to facilitate high-throughput analysis makes it an invaluable tool in the field of parasitology (Fink *et al.,* 2020) This has enabled the diagnosis of LF in both human and animal

reservoir blood, as well as in mosquitoes (Nuchprayoon, 2009). In order to develop PCR-based diagnostics with high sensitivity and specificity for diagnosis of LF, various target DNA repeats have been identified for detection of *W. bancrofti* and *B malayi*. For instance, DNA based PCR techniques have been developed for the identification of repetitive DNA sequences *Ssp I(ofr W. bancrofti)* and Hha *I (for B. malayi)* (Nuchprayoon, 2009). The oligonucleotide primers NV-1 and NV-2, which have been developed based on the Ssp I tandemly repeat sequence, have been widely used in PCR diagnostic studies of *W. bancrofti* (McCarthy *et al.,* 1996; McReynolds *et al.,* 1986).

2.4 Filarial Chitinase

Chitin is a homopolymer of poly-β (1-4)-linked N-acetylglucosamine monomer which forms part of the exoskeleton of arthropods and re1ported to be found also in some fungi and bacteria. Researchers narrate that chitin is the constituent of structures of nematode eggshell and eggshell-derivatives (Wu *et al.,* 2008). Chitinases are enzymes of glycosyltransferase family that catalyze the breakdown of chitin by hydrolysis. Is highly prevalent polysaccharide which primarily present in abundance in the cuticles, eggshells, microfilarial sheath and pharynx of nematode parasites (Adam *et al.,* 1996; Dravid *et al.,* 2015; Tachu *et al.,* 2008). The expression of chitinase is described to be localized in inner bodies of sheathed microfilariae of *W. bancrofti* and *B. malayi* from which the sheath comes direct from the eggshell. Identification and characterization of microfilaria chitinase protein as a component of *B. malayi* on the surface sheath was revealed by Canlas and his colleagues (Kennedy *et al.,* 2013). Expression of chitinase protein depends on developmental stages of the microfilaria. When the MF is intrauterine or newly born, chitinase protein is expressed in low quantity while expression increased dramatically when the MF enters the human host (Kennedy *et al.,* 2013; Quek *et al.,* 2022).

Filarial chitinases have been used by several researchers as diagnostic markers and vaccine candidates for in studying host immune response in LF infection (Kumar *et al.,* 2022)

2.5 The link between Filarial Infection and Other Parasitic Infections to HIV Infection

Concomitant parasitic infections have been reported to be associated with the risk of HIV infection and HIV/AIDS disease progression in Sub-Saharan Africa (Yegorov *et al.,* 2019) A cross sectional study done in Ethiopian migrants to Israel revealed a correlation between soil transmitted helminth and HIV infection (Bentwich *et al.,* 1996) . Immunologically, T-cells activation and T-helper-2 cells response following soil transmitted helminth reduce immune response and hence facilitate HIV acquisition (Chachage *et al.,* 2014; C). Several cross-sectional studies support the effect of parasites specifically helminths on HIV susceptibility (Gallagher *et al.,* 2005; Nielsen *et al.,* 2006, 2007). A cross-sectional study done in Zimbabwean women reported that women with genital schistosomiasis had almost three-times risk of HIV acquisition compared to those not infected with schistosomiasis (Kjetland *et al.,* 2006).

The primary factor contributing to increased HIV susceptibility rather than immunity based, is the disruption of mucosal barrier by eggs of *Schistosoma haematobium* (Brodish & Singh, 2016). With regards to lymphatic filariasis, the interaction between *W. bancrofti* and HIV have been examined by few studies , however majority of them have focused on lymphatic filariasis and HIV co-infection (Nielsen *et al.,* 2007; Simon, 2016; Talaat *et al.,* 2008, 2015). A study done by Nielsen *et al*., 2006 in North-eastern Tanzania revealed that individuals infected with *W. bancrofti* have higher HIV prevalence, although a follow up study of the same group did not find and evidence for the association of lymphatic filariasis and HIV (Nielsen *et al.,* 2006; Petersen *et al.,* 2009). In fact, among the numerous factors influence the risk of HIV infection, the major factor is behavioural (Jones *et al.,* 2018). A population based cohort study conducted by Kroidl *et al.,* 2016 in southwest of Tanzania, was the first study which described a significant increased risk of contracting HIV for lymphatic filariasis-infected people using CFA as marker for the diagnosis of LF(Kroidl *et al.,* 2016b). In their findings, teens and young adults group (14 to >25 years old) found to have high HIV risk compared to other age group (Kroidl *et al.,* 2016b). The reason for this evidence (Increased HIV infection risk by lymphatic filariasis) was further investigated by the same researchers in the same population and found that *Wuchereria bancrofti* cause host immune-activation by activating systemic

CD4 T cell which might be driving host susceptibility to HIV acquisition (Kroidl *et al.,* 2019). Several studies have demonstrated that activated CD4 T cells are major cellular reservoir for ongoing HIV replication in vitro (Card *et al.,* 2012; Corey *et al.,* 2015). Other study demonstrates that stimulation of monocytes by MF lysate induces a regulatory phenotype by expression of PD-L1 and IL-10 in vitro. The IL-10-dependant microfilaria-modulated monocytes are responsible for the suppress of T cell functions observed in LF infected individuals (O'Regan *et al.,* 2014).

The investigation of *Brugia malayi* microfilaria-specific proteins that form circulating immune complexes (ICs) play in IC-forming proteins in an immune evasion mechanism of the circulating microfilariae to avoid antibody mediated host immunity (Reamtong *et al.,* 2019). A study conducted by Nielsen and his colleagues in north-east Tanzania which aimed at assessing filarial specific cellular responsiveness reported asymptomatic participants who were MF positive had low levels of IL-10 compared with those who were CFA-positive and MF negative (Nielsen *et al.,* 2002).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

This study was conducted in Kyela. Kyelais one of the seven districts of Mbeya region in southern-highland of Tanzania. The district has population of approximately 249,261 people. It is bordered to the southeast by Lake Nyasa. Major economic activities of the indigenous people of Kyela are cultivation and fishing. The EMINI study was conducted at the National Institute for Medical Research (NIMR)-Mbeya Medical Research Centre (MMRC) between 2007 and 2011, as previously described (Kroidl *et al.*, 2016a).

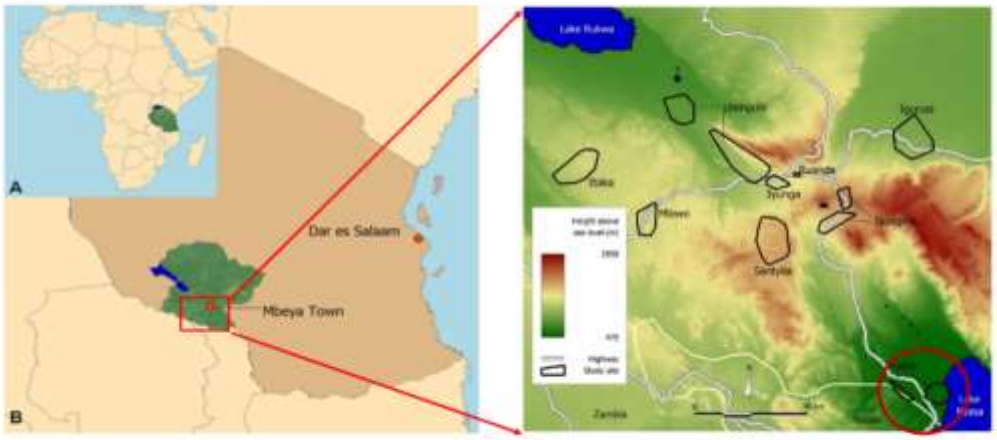


Figure 3.1: Study Site, Kyela District (Red Rectangle) Located Closer to Lake Nyasa

Source: (Kroidl et al., 2016a)

3.2 Study Design

This is a retrospective study that was conducted with 350 bio-banked blood samples collected from EMINI a previous study on evaluating and monitoring the impact of new interventions towards mitigating the effect of filariasis and soil transmission helminths. During EMINI study, various samples including blood were collected at interval of year for five years monitoring of MF both HIV status from 2007 to 2011. This study selected

396 bio-banked blood samples from individuals who tested positive for CFA but negative for HIV at baseline visit of the EMINI study in 2007.

3.3 Sampling Design

During each survey of the EMINI study, participants were visited and 40 ml of venous blood were drawn from each participant using anticoagulant tubes (CPDA, EDTA; BD Vacutainer). Fresh blood samples were processed within 6 hours of the blood draw at the National Institute for Medical Research- Mbeya Medical Research Centre (NIMR-MMRC) laboratories where the aliquots were made and stored at -20 °C freezers. Among the samples collected during EMINI study, non-random sampling technique was used to select 396 bio-archived blood samples which were CFA positive but HIV negative at survey one from adolescent and adults (≥ 14 years old) of the previous EMINI cohorts. Out of 396 obtained blood samples 350 were analyzed in this study while the rest (46) blood samples were dropped from analysis due to various reasons such as mislabeled, not from survey one and missing important data.

3.4 The Study Sample Size

The study done by Kroidl and her colleagues reported lymphatic filariasis prevalence of 23.8% (n=2104) at Kyela district (Kroidl *et al.,* 2016a). The minimum sample size for this study was calculated using the following formula:

$N = \{Z^2 * P (1 - P)\} / d^2$

Where; **N** is minimum sample size, **Z** is 1.962 (the z-score for a 95% confidence interval), and **P** is the prevalence of lymphatic filariasis, while $\mathbf{d^2}$ is the level of precision (5%)

Therefore:

$N = \{1.962 * 0.24(1-0.24)\} / 0.05^2$

N = 280.28, rounded down to 280.

In order to increase the likelihood of statistically significant differences among the analysed samples, we used all the CFA positive and HIV negative Biobanked blood

samples (396). A nonprobability, purposive sampling technique was used in selection of all bio-archived blood samples.

3.5 *Wuchereria bancrofti* Microfilaria RNA Isolation

Microfilaria RNA was isolated from the blood pellets using the Qiagen miRNeasy kit according to manufacture protocol. 5ml of TRIzol, a tissue lysing reagent, was added into 2ml of blood pellets then vigorously vortexed and homogenized using PreCellys beads and the homogenate left at room temperature for 5 min. Then 700 µl of 1-bromo-3-chloropropane was added into homogenate incubated for 10 min and centrifuged for 15 min at 12,000g at 4°C.

Upper aqueous phase was transferred into a new tube and RNA precipitated by adding 500 µl of absolute ethanol, then transferred into RNeasy min spin column and centrifuged at 8000 x g for 1min at 4°C. To remove all the DNA contaminates and ensuring that RNA is bound to the membrane, 80µl DNasel (10 µl DNase I stock solution to 70 µl Buffer RDD) was directly added into the RNeasy MiniSpin Column and incubated at room temperature for 15min to allow DNA digestion. The membrane was then washed twice by pipetting 500µl Buffer RPE into the RNeasy Mini spin column and centrifuge for 30s at ≥ 8000 x g (≥10,000 rpm). To remove the residue of wash buffer, RNeasy Mini spin column was centrifuged at full speed for 1 min. RNeasy Mini spin column was transferred to a new 1.5 ml RNA collection tube, 30 µl RNase-free water directly onto the RNeasy Mini spin column membrane and centrifuge for 1 min at≥8000 x g (≥10,000 rpm) to elute the extracted RNA, then stored at -80°C freezer (Majumdar *et al.,* 2015)

3.6 *Wuchereria bancrofti* Microfilaria RNA Analysis

Isolated RNA samples were treated with RNase-Free DNase (QIAGEN, Germany) during isolation the RNA integrity and concentration assessed using the Experion™ RNA HighSens Analysis kit (Bio-Rad Laboratories). One microliter (1 µL) of heat denatured RNA sample was loaded into the LabChip wells containing 9 µL of 1 % stained gel and loading buffer while 1µL of ladder loaded into a separate well without causing bubbles. The loaded chip was then placed in automated Experion electrophoresis station and the run carried out for 5 minutes.Thereafter the results of analysis were displayed.

3.7 Complementary DNA (cDNA) Synthesis

About 1.5 µg of isolated RNA was used to synthesized cDNA using the LunaScript ® Reverse Transcription kit (BioLabs). Briefly, 4 µL of the RT SuperMix and 5 µL of RNA sample plus 11 µL of nuclear free water were added into the reverse transcriptase (RT) PRC tubes to make a 20 µL reaction volume. Likewise, 4 µL of No RT control mix, 5 µL of RNA sample and 11 µL of nuclear free water were added into No RT PCR tubes as negative control for gDNA contamination. The cDNA synthesis took place at temperature profile of 25 °C for 2 minutes, 55 °C for 40 minutes and 95 °C for 1 minute for primer annealing, cDNA synthesis and heat inactivation respectively (Majumdar *et al.*, 2015).

3.8 *Wuchereria bancrofti* Microfilariae gDNA Isolation

Wuchereria bancrofti microfilariae gDNA were isolated from both the interphase and organic phase of the lysed and separated samples by TRIzol reagent and 1-bromo -3-chloropropane (ThermoFisher Scientific, Waltham, MA, USA). The DNA was precipitated with 700 µL of 100% ethanol per 1.5 ml of TRIZol reagent used for lysis after removing the aqueous phase underlying the interphase. The homogenate was then centrifuged at 2000x g at 4°C for 5 minutes. The pellet was resuspended in 1 ml of 0.1 M sodium citrate in 10% ethanol (pH 8.5) and then centrifuged for 5 minutes at 2000 RPM at 4°C for 5 min after 30 minutes incubation at room temperature (RT). This step was repeated twice to wash the DNA. Thereafter the pellets were air dried for 10 min and then resuspended in 600 µL of 8 mM NaOH to solubilize the DNA and centrifuged at 12000 x g at 4°C for 10 minutes. The supernatant was finally transferred into new tubes and stored at -20 °C. The quality and quantity of the isolated DNA was measured by using a NanoVue spectrophotometer (ThermoFisher Scientific). Where 1 µL of DNA sample was pipetted directly onto a novel sample plate, lower the sample plate head and the machine start to read the results and displayed on the screen in µL/ml.

3.9 Quality Control and Optimization of *W. bancrofti* Microfilariae Chitinase PCR

Primarily, the original aim was to quantify the chitinase gene from mRNA expressed by *W. bancrofti* microfilariae in blood samples. Discrete transcripts encode multiple chitinase isoforms in Brugian microfilariae and would have been a good way to

differentiate between microfilariae and mRNA from other stages (Arnold *et al.,* 1996). The established real-time PCR assay was very sensitive using frozen blood samples from Ghana, RNA extraction and real-time PCR protocols worked out using plasmid containing the sequence as positive control. However, the Tanzanian samples provided unsuitable RNA for analysis, we therefore carried on the analysis using the real-time PCR for chitinase for genomic DNA. Chitinase PCR assay was used for the amplification of target DNA and revealed the amplicons of the same size aligned with W. *bancrofti* MF chitinase plasmid (151 bp) used as a positive control

Three different sets of blood samples (known positive for *W. bancrofti*, known negative for *W. bancrofti* and known positive for other filarial worms) were used for the *W. bancrofti* microfilariae chitinase PCR sensitivity and specificity testing, one from West Africa one from Central Africa (Ghana, Cameron respectively) and one from East Africa, Tanzania the study area. All samples were parallel runed in triplicates. For the CFA and MF-positive Ghanaian selected as positive control samples, 6/6(100%) with >1700 MF/ml were positive for *W. bancrofti* MF chitinase. Another 30 CFA and MF negative lama blood samples from Ghana were used as negative controls, among those 29 of them (96.7%) were *W. bancrofti* MF chitinase negative.

In addition, 50 CFA negative samples from the EMINI study in Tanzania were PCR tested and they were all negative for *W. bancrofti* MF chitinase. Furthermore, specificity of the *W. bancrofti* microfilaria chitinase PCR was confirmed by testing blood samples of individuals infected with other filarial nematodes (from Cameroon): *M. perstans, L. loa,* and *O. volvulus*, which all tested negative, as shown in table 3.1

Table 3.1: Testing of Samples with Known Filarial Species and MF Count for Chitinase PCR Sensitivity and Specificity Measurement

Nematode species	N	CFA	*W. bancrofti MF count/µl*	MF PCR pos	MF PCR neg
W. bancrofti	1	pos	1740	1	0
W. bancrofti	1	pos	2030	1	0
W. bancrofti	1	pos	2170	1	0
W. bancrofti	1	pos	2420	1	0
W. bancrofti	1	pos	4090	1	0
W. bancrofti	1	pos	4360	1	0
Mansonella perstans	20	neg	NA	0	20
Loa loa	6	neg	NA	0	6
Onchocerca volvulus	20	neg	NA	0	20
*not infected**	30	neg	0	1	29
not infected[#]	50	neg	Not determined	0	50

N= count, CFA = circulating filarial antigen, *****samples from a Wb endemic area, tested for CFA and MF, [#]samples from the Tanzanian study village, tested for CFA

3.10 qPCR for the Detection of *W. bancrofti* Microfilaria Specific Chitinase Gene

Briefly, a 151 bp fragment of the target cDNA templet of *W. bancrofti* chitinase gene (GenBank: AF250997.1) was amplified in QuantiNova Probe PCR master mix (QIAGEN, Hilden, Germany) by using 5'- AAACAGCGATTGGAGCAGCG-3' and 5'-ACCATGTACACCCCGACACC-3' forward and reverse primers (Microsynth, Balgach, Switzerland) respectively with concentration of 10 nM and 75 µM chitinase TaqMan 6-Fam labeled probe (Biomers.net GmbH, Ulm, Germany) in a total reaction volume of 20 µL. The positive control for this experiment involved the use o of plasmid DNA containing the *W. bancrofti* endochitinase sequence. On the other hand, the no template control was established using nuclease- free water. The amplification and detection process were carried out under the following optimal conditions; 95℃ for 15 minutes as initial denaturation step, followed by 45 cycles of 95°C for 10 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 20 seconds using a RotorGene 6000 thermocycler (Corbett Research, Sydney, Australia). For the analysis using Rotor-Gene Q Software (version 2.3.1.49, Qiagen), the threshold was established at 0.02 and outlier removal at 15%. Samples with Ct value ≤32 was deined as positive while those with Ct values greater than 32 was defined as negative. The internal control of human beta actin

(Gen- Bank: NG_007992.1) was amplified using a reaction volume of 20 µL of 1x HotStar Taq master mix (Qiagen, Hilden, Germany). With the final concentration of 10 M forward (50-GAT GAG ATT GGC ATG GCT TTA-30) and 10 M reverse (50-AAC CGA CTGCTG GTG TCA CCT TC-30) primers (Microsynth, Göttingen, Germany), 200 _M dNTPs,1_ SYBR Green (1:1000 diluted in DMSO, Roche, Mannheim, Germany), and 0.5 units of HotStar Taq polymerase in a RotorGene 6000 (Corbett Research, Cambridge, UK) thermocycler under the following conditions: 95 °C for 15 min as the initial denaturation step, followed by 45 cycles of 94 °C for 10 s, annealing at 58 °C for 20 second and extension at 72 °C for 20 second with acquisition on the green (FAM) channel (Albers *et al.*, 2014). To ensure specific amplification, a melting curve was conducted from 72 °C to 95 °C with a 1°C increase per step and a 4 second interval between steps. Data was acquired on the green (FAM) channel and analyzed using Rotor Gene Q software (ver. 2.3.1.49, Qiagen) at the threshold of 0.3. All DNA samples tested positive for human beta-actin PCR which implies that DNA extraction procedure was efficient and valid. To eliminate the possibility of PCR inhibitors in the template DNA, a mouse interferon gamma (mIFN-y) PCR was performed on chitinase negative samples. The results of all mouse interferon gamma (mIFN-y) PCR runs indicated the absence of PCR inhibitors in the isolated DNA.

3.11 Ethical Clearance and Approval

The blood samples were collected during the EMINI study, which was approved by the Tanzanian National Health Research Ethics Committee (NIMR/HQ/R.8a/Vol.IX/358). Permission to use these previously collected human blood samples was granted by the Mbeya Medical Research Ethics Committee (GB.152/377/01/194 and SZEC-2439/R. A/V.1/41), the Tanzanian National Health Research Ethics Committee (NIMR/HQ/R.8a/Vol. IX/2856) and the Ethics Committee of the Medical Faculty of the University of Munich (project ID: 18-377).

3.12 Data Collection and Management

3.12.1 Data Collection

All the information generated during EMINI study questionnaire including demographic data such as age, sex, marital status, number of sex partners, use of condoms and social economic status plus HIV status were included in this study to carry out the multivariate analysis. PCR results obtained from gDNA were analyzed by using the Rotor Gene^{TM}6000 analysis software and data were transcribed into excel for statistical analysis. Participants were categorized into gender, age group, and other HIV confounding factors such as marital status, number of sex partners, circumcision and use of condoms.

3.12.2 Statistical Analysis

Statistical analysis was done using STATA 14 (StataCorp, College Station, TX, USA) and GraphPad Prism software, version 8 (GraphPad Software Inc., La Jolla, CA, USA). To compare binary outcomes between groups and the chitinase positive the Pearson's chi-square test was used. The analysis of *W. bancrofti* MF chitinase positive and the incidence of HIV was done by univariable and multivariable log link binary regression to determine the association between the variables and outcome. To investigate the potential confounding effects of other established risk factors for HIV (eg sex, marital status living with discordant partner etc), Non-adjusted correlation between the incidence of HIV and each of these risk factors was evaluated to classify possible variable separately for age group in order to potential differences in these associations at different age. In additional we run binomial regression to determine the correlation between chitinase positive and LF positive from the previous cohort.

CHAPTER FOUR

RESULTS

4.1 Description of Study Participants

Cryopreserved blood samples collected from peoples in Kyela district who had previously participated in EMINI cohort study were used for the additional analysis in current study. Participants of all age were enrolled during the EMINI study in 2007, and they were annually followed up for five years. Blood sample was collected from study participants during each follow up visit and tested for HIV and other infections. Socioeconomic information about the study participants, past and present medical histories as well as personal knowledge of various diseases were also recorded in a questionnaire. The current study used available 350 of 396 CFA positive biobanked samples of HIV negative individuals for the detection of *W. bancrofti chitinase* microfilaria (Table 4.1). Study participants' ages ranged from 14 to 90 years with a median age of 32.7 years (IQR: 21.8-57 years), and 170 (48.6%) were female. At the time of enrolment and sample collection, all participants had HIV negative baseline tests.

Table 4.1: Description Information of Study Participants

Characteristic	Total N(%)	Female, N (%)	Males, N (%)	P Value
All (CFA+ HIV-)	350	170 (48.6)	180(51.4)	
Age group (years)				
14 <25.	117 (33.4)	72(42.4)	45 (25.0)	0.07
25 – <45.	110(31.4)	54(31.8)	56 (31.1)	
>45.	123(35.2)	44(25.8)	79(43.9)	
Median age in years (IQR)	21.5 (35-88)	31.8(21.7-56.8)	32.7(21,6-50.8)	
SES, median (IQR)	3.1 (0.9-5.8)	3.0(0.8-5.6)	3.2 (1.0-6.1)	0.5

Key: *p-value was calculated using Chi-square for binary variable (sex, age group) and Mann-Whitney test for continuous variable (age, SES). $IQR: Interquartile range

4.2 *Wuchereria bancrofti* Microfilaria Chitinase PCR

Out of 350 blood samples that were analysed, 12 (3.4%) were positive for *W. bancrofti* microfilaria chitinase (figure 4.1) among them 9/350 (2.6%) samples tested positive for female participants and 3/350 (0.9%) for male individuals (p=0.062). There was no

difference in proportion of *W. bancrofti* chitinase across different age groups when the cohort was stratified according to age. Among young individuals aged 14 to <25 years, five out of 350 (1.4%), three out of 350 (0.9%) among individuals aged 25 to <45 and four out of 350 (1.1%) for adults aged >45 years were positive for *W. bancrofti* chitinase (p=0.803, Figure 4.1).

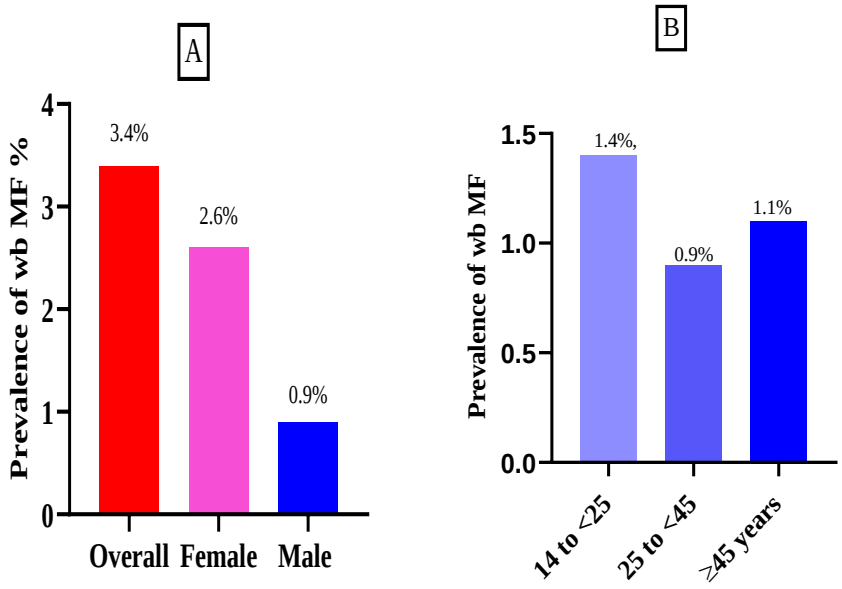
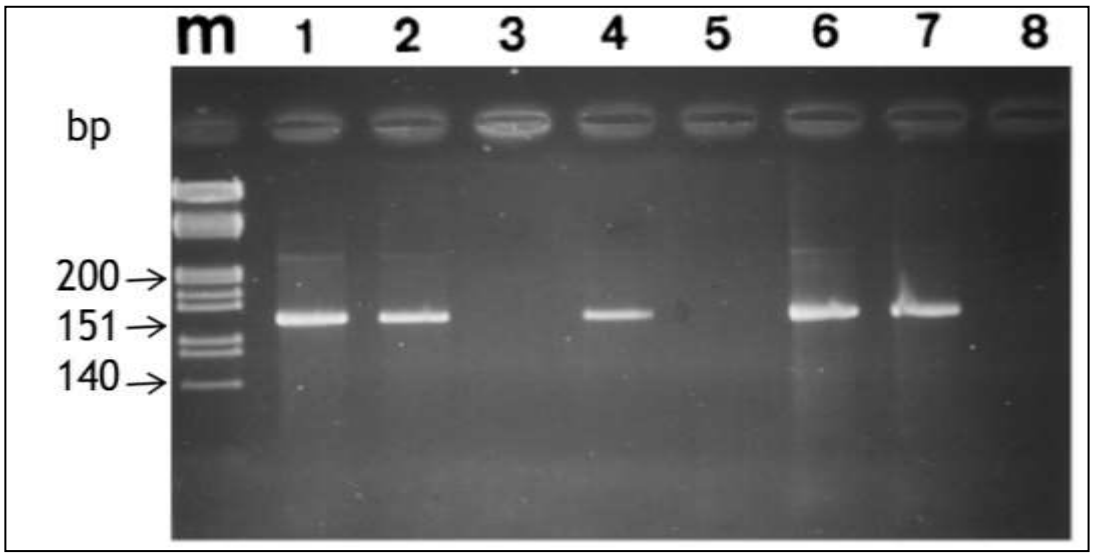


Figure 4.1: Prevalence of *W. bancrofti* MF Chitinase Stratified by Gender(A) and by Age Groups (B)

The amplified target DNA of *Wuchereria bancrofti* microfilaria-chitinase was observed running parallel with the ladder and *W. bancrofti MF chitinase* plasmid positive controland displayed single amplicons of 151bp equivalent to the loaded ladder. Figure 4.2



Base pair (bp), columns 1: DNA ladder; columns 1 – 6: Wb gDNA samples displaying single amplicons of 150 bp; column 7, Wb MF chitinase plasmid positive control; column 8: no template control.

Figure 4.2: *W. bancrofti* MF Chitinase PCR Amplification Results Showing Bands of the Ladder and Target DNA (151 bp) and Controls

4.3 HIV Incidence in *Wuchereria bancrofti* Microfilariae-Positive Individuals

During the five-year follow up, 22 (5.71%) out of 350 CFA positive individuals who were HIV negative during the enrollment became HIV positive. Among them, 8(2.29%), 7(2%), 3(0.86%) and 4(1.14%) individuals seroconverted to HIV positive during 2008, 2009, 2010 and 2011 years respectively. Three out of 22 individuals who seroconverted were microfilaria positive (Table 4.3).

Table 4.3: Individuals Seroconverted During the Course of FiveYears of Follow-Up

Study year	CFA Positive	HIV Positive, n (%)	MF Positive, n (%)
2007	350	0(0)	0(0)
2008	350	8(2.29)	1(0.29)
2009	350	7(2)	1(0.29)
2010	350	3(0.86)	0(0)
2011	350	4(1.14)	1(0.29)
Total	**1400**	**22(5.71)**	**3(0.86)**

The incidence of HIV infections among microfilaremia individuals during the course of four-year follow-up period was 1.98 per 100-person years (PY). The 4.58 times higher incidence of HIV was observed among the *W. bancrofti* MF chitinase positive individuals compared to the *W. bancrofti*-infected but MF negative individuals after using a multivariable analysis when adjusted for age, sex and socio-economic status (p=0.014, Table 4.4).

Table 4.4: Uni- and Multivariable Analysis Showing the Association between HIV and MF Chitinase Gene Positivity among *W. bancrofti*-Infected Individuals

				Univariable			Multivariable		
Covariate	**Exp.**	**N-pos**	**Incidence per100 PY**	**IRR**	**95% CI**	**P-value**	**IRR.**	**95% CI**	**P-value**
All	1109	22	1.98						
MF at survey 1									
Neg*	1,070	19	1.78	1.00			1.00		
Pos	39	3	7.75	**4.29**	**(1.33 to 13.9)**	**0.015**	**4.58**	**(1.37 to 15.4)**	**0.014**
Gender									
Female*	537	11	2.05	1.00	- -	-	1.00	-	-
Male	572	11	1.92	0.94	(0.41 to 2.18)	0.885	0.90	(0.39 to 2.07)	0.796
Age									
14 - <25*	312	6	1.92	1.00	-	-	1.00	-	-
25 - <45	338	10	2.61	1.35	(0.49 to 3.75)	0.560	1.46	(0.54 to 3.95)	0.453
>=45	413	6	1.45	0.76	(0.25 to 2.36)	0.636	0.75	(0.25 to 2.12)	0.530
SES rank (per unit)	-	-	-	0.91	(0.78 to 1.06)	0.213	0.89	(0.76 to 1.04)	0.134

Key: *Exp. = exposure time in person years; N-pos = number of positives; PY = person years IRR = Incidence Rate Ratio; 95% CI =95% confidence interval; * reference stratum*

Individuals from the previous EMINI study who were not infected with *W. bancrofti* (CFA negative) were included in the multivariable analysis. Due to the fact that microfilariae are the offsprings of adults filarial worm, can only be detected from

individual who habor the adult worms which confirmed by CFA positivivity, this raise an assumption that all CFA negative would be MF negative. To ensure this assumption, all the 50 CFA negative samples from Tanzania were indeed PCR negative.

There was no significant difference in risk of HIV acquisition among maried and never maried individuals who were MF positive (p value 0.922 and 0.772 respectively). The multivariable analysis revealed that HIV infection incidence is two times as higher in *W. bancrofti* positive MF positive participants than as in CFA negative subjects. In addition, individuals with *W. bancrofti*-positive-MF showed a 12 times HIV incidence higher than that of CFA negative people (Table 4.5)

Table 4.5: Uni- and Multivariable Analysis Showing the Association between HIV and LF Status (CFA and MF Combined) when Adjusted for Commonly Known HIV Risk Factors

				Univar able			Multivariable		
Covariate	**Exp.**	**N-pos**	**IR per 100 PY**	**IRR**	**95% CI**	**P-value**	**IRR**	**95% CI**	**P-value**
All	3,187	38	1.19						
Gender									
Female*	1,642	22	1.34	1.00	- -	-	1.00	- -	-
Male	1,546	16	1.04	0.77	(0.41 to 1.48)	0.436	0.55	(0.42 to 1.24)	0.148
Age									
14 - <25*	1,337	10	0.75	1.00	- -	- -	1.00		
25 - <45	964	18	1.87	2.48	(1.14 to 5.39)	0.022	1.73	(0.66 to 4. 52)	0.263
>=45	886	10	1.13	1.51	(0.63 to 3.62)	0.361	1.27	(0.45 to 3.59)	0.657
LF Status									
CFA -*	2,101	16	0.76	1.00	- -	-	1.00	- -	-
CFA+.MF-	1,051	19	1.81	**2.36**	**(1.21 to 4.59)**	**0.012**	**2.12**	**(1.71 to 4.20)**	**0.030**
CFA+.MF+	35	3	8.69	**11.1**	**(3.43 to 36.2)**	**<0.001**	**12.7**	**(3.48 to 46.2)**	**<0.001**
Marital Status									
Never married*	1,113	7	0.63	1.00	- -	-	1.00	- -	-
Married	1,357	19	1.40	2.22	(0.93 to 5.29)	0.073	0.94	(0.27 to 3.22)	0.922
Separated[#]	718	12	1.67	2.64	(1.04 to 6.73)	0.042	1.20	(0.35 to 4.10)	0.772
No. of sex partners									
None*	285	2	0.70	1.00	- -	-	1.00	- -	-
One	1,487	20	1.35	1.91	(0.44 to 8.21)	0.386	2.63	(0.51 to 13.7)	0.251
Two or more	378	11	2.91	4.09	(0.90 to 18.6)	0.069	8.52	(1.49 to 48.7)	0.016
No info	1,038	5	0.48	0.69	(0.13 to 3.56)	0.656	1.29	(0.24 to 7.01)	0.771
Circumcised (Male)									
No*	1,351	15	1.11	1.00	- -	-	1.00	- -	-
Yes	195	1	0.51	0.42	(0.06 to 3,19)	0.418	0.48	(0.06 to 3.60)	0.476
N/A (female)	1,642	22	1.34	1.00	(omitted)	-	1.00	(omitted)	-
SES rank (per unit)	-	-	-	0.92	(0.81 to 1.03)	0.158	0.91	(0.80 to 1.03)	0.146

Key: *Exp. = exposure time in person years; N-pos = number of positives; PY = person years IRR = Incidence Rate Ratio; 95% CI =95% confidence interval; - =negative; + = positive; * reference stratum.*

To compare the HIV incidence between men and women, the multivariable analysis demonstrated that women had higher HIV incidence compared to men, uncircumcised had high HIV incidence compared to circumcised, the same applied to individuals with multiple sex partners (Table 4.5).

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Using *W. bancrofti* microfilariae chitinase PCR test on 350 circulating filarial antigenemia positive (CFA-positive) bio-archived blood samples, this study showed a prevalence of microfilariae to be 3.4%. The prevalence rate of microfilaria in this study is comparable to the study Fimbo and his colleagues in Northern Tanzania which reported the prevalence of 5.2% (11 out of 211) CFA positive individuals (Fimbo *et al.,* 2020a). Given the fact that this study was carried out in a region where lymphatic filariasis was endemic at the time of sample collection and the MDA was not yet launched, this rate is significantly lower than expected. However, because the initial large study was planned for other purpose, samples were collected during the day, which could significantly reduce the amount of MF in the peripheral blood due to nocturnal periodicity of MF. Additionally, the same individuals in the study area were given ivermectin for the treatment and control of *O. volvulus,* another filarial specie in the year before samples collection. (Mehta *et al.,* 2018; Aboagye-Antwi *et al.,* 2015; Simonsen *et al.,* 2014; Weil *et al.,* 2008). The effect of ivermectin treatment for onchocerciasis had been reported to reduce the prevalence of MF but not the overall *W. Bancrofti* prevalence through CFA testing in Ethiopians as similarly reported in Tanzania (Endeshaw *et al.,* 2015; Iboh *et al.,* 2012). Microscopic examination of blood for microfilaria wasn't done during the previous study, therefore the association of LF and HIV wasn't determined by latent (microfilaremia) or patent infection (amicrofilaremia). Therefore, this study strived to fill this gap by retrospectively assessing microfilaria status in archived blood samples by PCR method.

This study indicates that microfilaremia is significantly associated with HIV acquisition by 4.5-fold increase the risk of HIV incidence in individuals who are positive for *W. bancrofti* microfilaria chitinase as compared to *W. bancrofti* microfilaria chitinase negative individuals. This finding doubled that previously reported by Kroidl and colleagues who observed a 2.3-fold increased risk of HIV susceptibility in individuals with CFA positive than those who were CFA negative from the same cohort (Kroidl et

al., 2016b). The HIV incidence of 1.98 per 100 person-year was observed in 22 filarial-infected individuals during the four years follow-up, while the incidence of HIV among filarial-infected and microfilaria positive individuals was 7.75 per 100 person-year versus 1.78 per 100 person-year in filarial-infected but microfilaria negative individuals (p=0.014). Incidence of HIV in microfilaremic participants in this study shows a 6-fold higher when compared to the previous reported of 1.91 per 100 person-year in persons with circulating antigenemia (Kroidl *et al.,* 2016b). High incidence rate of HIV in microfilaremic individuals might be due to the fact that microfilaremia is associated with impaired immune response as it has been revealed that microfilaremic individuals exhibited elevated levels of IgE, while simultaneously showing decreased concentrations of IL-6, IL-10, IFN-gamma, and TNF-alpha in their blood, in contrast to those who were infected with filaria but did not present microfilaremia(Arndtset *al.,* 2012).

It is apparently that microfilariae, the offsprings of adult filarial worm are only released by the individual infected with adult worms. Based on that knowlegde we included the data of known CFA- negative participants from the same stady area in to the analysis. These CFA negative participants they shoul be amicrofilaremic in case the initial CFA test revealed a true negative result. To verify this, we performed a *W.bancrofti* chitinase PCR on 50 randomly selected CFA negative blood samples from the study cohort and discovered that all results were negative. To count for potential confounding, known risk factors for HIV transmision were also included in the analysis. The multivariable analysis showed an increased susceptibility to HIV in people with more than two sex partners and a protective effect of male circumcision, confirming the validity of the analyzed data. When compared these two groups, microfilaremia Wb-uninfected individuals demonstrated a 12.7 times increased risk for HIV than the amicrofilaremia *W. bancrofti*-infected individuals who showed a 2.1 times risk to acquire HIV infection (p<0.001). We hypothesize that the immunocompromised state of microfilaremia *W. bancrofti* as described by Arndts *et al.,* it has been shown to decrease antiviral ability and increase the risk of transmitting viral infections, particularly HIV in this case specifically. This hypothesis is supported by a recent study from Togo which demonstrated that hookworm and other nematodes infected persons have higher HPV viral load (Omondi *et al.,* 2022).

The previous study had some logistical challenges, one was blood samples being collected during the day as of which the significance number of microfilariae are not found in the peripheral blood due their nocturnal periodicity characteristic (Dreyer *et al.,* 2007), this could account for the low number of MF positive PCR test results. Dreyer et al., also reported that microfilaremia levels peak between 23:00h and 01:00h and decline to the lowest levels between 12:00h and 15:00h (Dreyer *et al.,* 2007). Hence, a very low levels of MF counts might not have been able to be detected by the established *W. bancrofti* chitinase PCR method. Indeed, the lowest MF number (200mf/ml) positive control samples (CFA+MF+) revealed negative qPCR results.

It was hypothesized that the 12 individuals who tested positive for MF using PCR represent merely "the tip of the iceberg" indicating those with the highest MF load; however, this remains unverified. Nonetheless, despite the limited number of MF positive cases a significant heightened susceptibility to HIV among the filarial-infected participants who were MF positive was distinctly observed. The adjustment of the analysis for established HIV risk factors to avoid confounding did not alter this outcome. However, whether the previously reported rise in HIV incidence among individuals infected with *Wuchereria bancrofti* was solely attributable to the MF-positive subgroup, or if both the adult worms and their offspring play a role in this association could not be ascertained.

5.3 Study Limitations

This study has some limitations: In this study we had to use a different definition of "helminth positive" rather than that used in the previous published article (Kroidl *et al.,* 2016a). In previous study, the risk of new HIV infections was determined by time-period of the stable *W. bancrofti* infection. As the adult filarial worm is resistant to either ivermectin and albendazole it is possible to be detectable over several years. Therefore, the annually detection of CFA for five consecutive years was established. Filarial control program through mass drug administration was started by the government during the EMINI study, at this time span the MF were not useful due to the fact that MF are sensitive to ivermectin a primary drug used in MDA. In this particular study we measured MF in the blood only on one time point (the first survey of the previous study in 2007). The low

frequency of *W. bancrofti* MF chitinase positivity is another limitation. Initially, the assumption was at least 50% percent of the blood samples of *W. bancrofti*-infected individuals would be positive for MF. In fact, the low prevalence of *W. bancrofti* MF chitinase, 3.4% (12/350) was an unexpected outcome.

5.2 Conclusion

- This study showed prevalence of microfilaria of 3.4% which looks low than expected from CFA positive samples, this might be due to reason that the blood samples were collected during the daytime.
- Microfilaremia is significantly increases an individual's susceptibility to HIV in *W. bancrofti*-infected individuals. This subgroup of *W. bancrofti*-infected people has higher HIV susceptibility than the larger cohort of all *W. bancrofti*-infected participants (regardless of MF status), which had a 2-fold increase risk for HIV.
- The risk of contracting HIV is roughly 10-12 times higher in *W. bancrofti*-infected individuals than uninfected ones. When comparing the two subgroup of *W. bancrofti*-infected individuals, the association with HIV incidence is 4.58 times higher in patent filarial infection (microfilaremic) than the latent (amicrofilaremic) filarial infection.

5.5 Recommendations

Strengthen and sustain the mass drugs administration (MDA) Programme and emphasize on anti-filarial therapy in LF and HIV co-endemic areas can lower the incidence of HIV in filarial infected individuals.

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APPENDICES

Appendix I: Publication

pathogens

MDPI

Article

Increased HIV Incidence in *Wuchereria bancrofti* Microfilaria Positive Individuals in Tanzania

Jonathan Mnkai [1,†], Manuel Ritter [2,†], Lucas Maganga [1], Leonard Maboko [1,3], Willyhelmina Olomi [1], Petra Clowes [1], Jessica Minich [2], Agola Eric Lelo [4], Daniel Kariuki [5], Alexander Yaw Debrah [6], Christof Geldmacher [7,8], Michael Hoelscher [7,8], Elmar Saathoff [7,8], Mkunde Chachage [1,9], Kenneth Pfarr [2,10,‡], Achim Hoerauf [2,10,11,‡] and Inge Kroidl [7,8,*,‡]

1 National Institute of Medical Research (NIMR), Mbeya Medical Research Center (MMRC), Mbeya P.O. Box 2410, Tanzania
2 Institute for Medical Microbiology, Immunology and Parasitology (IMMIP), University Hospital Bonn (UKB), 53127 Bonn, Germany
3 Tanzania Commission for AIDS, Dodoma P.O. Box 2904, Tanzania
4 Kenya Medical Research Institute (KEMRI), KNH, Nairobi, Kenya
5 College of Health Sciences, Jomo Kenyatta University of Agriculture and Technology (JKUAT), Juja, Kenya
6 Kumasi Centre for Collaborative Research (KCCR), Kwame Nkrumah University of Science and Technology, UPO, PMB, Kumasi, Ghana
7 Division of Infectious Diseases and Tropical Medicine, University Hospital of the University of Munich (LMU), 80802 Munich, Germany
8 German Centre for Infection Research (DZIF), Partner Site Munich, 80802 Munich, Germany
9 Mbeya College of Health and Allied Sciences (UDSM-MCHAS), University of Dar es Salaam, Mbeya 608, Tanzania
10 German Centre for Infection Research (DZIF), Partner Site Bonn-Cologne, 53127 Bonn, Germany
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† These authors contributed equally to this work.
‡ These authors contributed equally to this work.

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Citation: Mnkai, J.; Ritter, M.; Maganga, L.; Maboko, L.; Olomi, W.; Clowes, P.; Minich, J.; Lelo, A.E.; Kariuki, D.; Debrah, A.Y.; et al. Increased HIV Incidence in *Wuchereria bancrofti* Microfilaria Positive Individuals in Tanzania. *Pathogens* **2023**, *12*, 387. https://doi.org/10.3390/pathogens12030387

Academic Editors: Anna Rosa Garbuglia and Lawrence S. Young

Received: 13 December 2022
Revised: 23 February 2023
Accepted: 24 February 2023
Published: 28 February 2023

Abstract: Background: Infections with *Wuchereria bancrofti* are associated with reduced immunity against concomitant infections. Indeed, our previous study described a 2.3-fold increased HIV incidence among individuals with *W. bancrofti* infection, as measured by the circulating filarial antigen of the adult worm. This new study aimed to retrospectively determine microfilariae status of the participants to assess if the previously described increased HIV susceptibility was associated with the presence of MF in the same cohort. Methods: CFA positive but HIV negative biobanked human blood samples ($n = 350$) were analyzed for *W. bancrofti MF* chitinase using real time PCR. Results: The PCR provided a positive signal in 12/350 (3.4%) samples. During four years of follow-up (1109 person years (PY)), 22 study participants acquired an HIV infection. In 39 PY of *W. bancrofti* MF chitinase positive individuals, three new HIV infections occurred (7.8 cases per 100 PY), in contrast to 19 seroconversions in 1070 PY of *W. bancrofti* MF chitinase negative individuals (1.8 cases per 100 PY, $p = 0.014$). Conclusions: In the subgroup of MF-producing Wb-infected individuals, the HIV incidence exceeded the previously described moderate increased risk for HIV seen in all Wb-infected individuals (regardless of MF status) compared with uninfected persons from the same area.

Keywords: HIV; incidence; lymphatic filariasis; microfilariae; immunomodulation

1. Introduction

Lymphatic filariasis (LF) is a neglected parasitic tropical disease that affects the lymphatic system and can result in lymphedema (elephantiasis) and edema of the scrotum (hydrocele). LF is a leading cause of long-term and permanent disability in the world [1]. The numbers of infected individuals have dropped during the last two decades due to large

Appendix II: Ethical Approval

UNITED REPUBLIC OF TANZANIA
MINISTRY OF HEALTH, COMMUNITY DEVELOPMENT, GENDER, ELDERLY AND CHILDREN

Cable ReferralHospital
Telephone: 2503456/2503351
Fax 2503577
Email: info@mzrh.go.tz

MBEYA ZONAL REFERRAL HOSPITAL
P. O. BOX 419
MBEYA

24th October 2019

Ref No: SZEC-2439/R.A/V.1/41

Mr. Jonathan Mnkai,
NIMR-MMRC,
P.O. Box 2410,
Mbeya– Tanzania.

RE: **Request for Ethical Clearance for Protocol "Prevalence of *Wuchereriabancrofti* microfilariae and its association with HIV in blood samples from people living in Kyela District".**

Reference is made for the aforementioned subject.

The Mbeya Medical Research and Ethics review Committee received and reviewed your protocol during a meeting held on 22nd October 2019. I would like to inform you that, the Committee has granted Ethical Clearance of the above mentioned study protocol for the period of one year, from **22nd October 2019** to**21st October2020**.

This Ethical Clearance approval bears the following specifications;

1. If samples will be shipped outside the country, a Material Transfer Agreement (MTA) will need to be finalized;
2. To comply with approved study protocol procedures, and at all times you are responsible for ethical conducts of your research;
3. You must notify the Mbeya Medical Research and Ethics Committee in writing regarding any alteration or deviation to the protocol;
4. To submit progress reports to the Committee every six months;
5. If the research has been completed, abandoned, discontinued or not completed for any reason you are required to submit a final report to the Committee;
6. Approval is given for one year from **22nd October 2019** to**21st October 2020**, and if you are unable to complete your research within the one year validation period you will be required to write to Mbeya Medical Research and Ethics Committee to request for an extension;
7. You must notify immediately the Mbeya Medical Research and Ethics Committee of any adverse event and/or unforeseen events that might affect continued ethical acceptability of the research.

Sincerely,

Executive Director
Mbeya Zonal Consultant Hospital
P.O. Box 419
Mbeya, Tanzania

.....
Dr.Godlove F. Mbwanji
Chairman
Mbeya Medical Research and Ethics review Committee.

cc: The Chairman; National Health Research Ethics Review Committee; P.O.Box 9653, Dar es Salaam.
cc:RMO - Mbeya.

All Communication should be addressed to the Chairman, Mbeya Medical Research and Ethics Committee

Appendix III: Research Proposal Approval Letter

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY
DEAN
MAR
SIGN
SCHOOL OF BIOMEDICAL SCIENCES

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
DIRECTOR, BOARD
OF POSTGRADUATE STUDIES

P.O. BOX 62000
NAIROBI – 00200
KENYA
Email: director@bps.jkuat.ac.ke TEL: 254-67-5870000/1-5

REF: JKU/2/11/TM305-0797/2018 **04TH MARCH, 2021**

MNKAI JONATHAN LEWIS
C/o SOBMS
JKUAT

Dear Mr. Lewis,

RE: APPROVAL OF RESEARCH PROPOSAL AND APPOINTMENT OF SUPERVISORS

Kindly note that your MSc. research proposal entitled: **"PREVALENCE OF *Wuchereria bancrofti* MICROFILARIE AND ITS ASSOCIATION WITH HIV IN BLOOD SAMPLES FROM PEOPLE LIVING IN KYELA DISTRICT"** has been approved. The following are your approved supervisors:-

1. Dr. Eric Lelo
2. Prof. Daniel W. Kariuki
3. Dr. Mkunde Chachage

Yours sincerely,

PROF. LOSENGE TUROOP
DIRECTOR, BOARD OF POSTGRADUATE STUDIES
Copy to: Dean, SOBMS
/cm

Appendix IV: Admission Letter

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
P.O. BOX 62000, CITY SQUARE, NAIROBI, 00200, KENYA. TELEPHONE: (067) 52711, MOBILE NO. 0708-602-225
FAX: (067) 52446, THIKA
Office of the Director (BPS)
E-mail: director@bps.jkuat.ac.ke

TM305-0797/2018 **Date: 08th June, 2018**

Mnkai Jonathan Lewis
P.O. Box 2410 Mbeya, TanzaniaDear Applicant,

RE: OFFER OF ADMISSION FOR M.Sc. DEGREE COURSE

Following your application, I am pleased to inform you that you have been offered admission for M.Sc. in **Molecular Medicine** in the Institute of Tropical Medicine and Infectious Diseases (ITROMID) at JKUAT. **Kindly note that this admission is valid for two (2) years after which the applicant will be required to re-apply.**

The programme will be based at the Kenya Medicine Research Institute (KEMRI). Your programme coordinator will be Director, Institute of Tropical Medicine and Infectious Diseases (ITROMID).

The offer of admission does not include any funding and all fees and other charges will be met by yourself or your sponsor. You should also note that registration for the programme would only be possible on payment of the requisite fees and upon verification of your **Original certificates** and **National ID Card/Passport**.

Fees should be paid through:

1. **K.C.B, Account Number 1104167611,Account Name, ITROMID, Branch- KIPANDE**

The first semester of the course began on **08th May, 2018.** You are expected to register not later than two weeks after the date of the registration. No registration will be accepted after the said period. **The registration will be conducted at KEMRI**

Enclosed please find copies of the following:
a) Fees schedule
b) Acceptance/Registration Forms
c) Medical Examination Form (JKU/6)

Feel free to contact the Coordinator Graduate Programmes, KEMRI or the undersigned if you need further clarification on any matter regarding the above issues.

PROF. MATHEW KINYANJUI
DIRECTOR, BOARD OF POSTGRADUATE STUDIES
Copy to:
Registrar, AA
Director, ITROMID
Coordinator Graduate Programmes, KEMRI
Encl

✓ to collect