EPIDEMIOLOGY AND HOST GENETIC FACTORS ASSOCIATED WITH
BANCROFTIAN FILARIASIS IN ENDEMIC COMMUNITIES OF NORTH
EASTERN TANZANIA

HAPPINESS JEREMIA MSHANA

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF PHILOSOPHY OF SOKOINE UNIVERSITY OF
AGRICULTURE. MOROGORO, TANZANIA.

2017
EXTENDED ABSTRACT

Tanzania started a countrywide lymphatic filariasis elimination programme in 2000 adopting the mass drug administration (MDA) strategy. The drugs used for the MDA programme are a combination of ivermectin and albendazole. Clinically, the initial stages of lymphatic filariasis present as an acute febrile conditions with adenolymphangitis (ADL). Later on the disease is characterized by development of scrotal swelling (hydrocele) and swelling of the legs (lymphoedema) which gradually lead to elephantoid oedema. There is limited information on the current epidemiological trend of infections in areas where MDA implementation is ongoing and the immunological markers for early diagnosis of the disease have not been identified. The present study aimed at assessing the current status of bancroftian filariasis infection rate and morbidity in areas where MDA has been administered for over eight rounds as well as the clinical disease presentation and possible human genetic factors associated with disease development. The study was a cross-sectional descriptive study involving 472 individuals (>18 years) from endemic communities in Tanga region in north-eastern Tanzania where MDA has been implemented. Clinical data, socio-demographic survey and circulating filarial antigen (CFA) test was conducted using questionnaire and immuno chromatographic card test according to the manufacturer’s instructions respectively. A total of 76 individuals were tested for the presence or absence of a 23 nucleotide deletion within the 5’ untranslated region of toll like receptor 2 using allele specific real time -polymerase chain reaction (qPCR). A total of 472 individuals were screened: 307/472 (65.1%) of which were males while 165/472 (34.9%) were females. Of those, 272 were recruited for the study of which 87.86% were males and 12.14% were females. The proportion of CFA was 5.51%, that of hydrocoele was 73.2%, and that of lymphoedema was 16.0%. The proportion of hydrocoele combined with lymphoedema was 5.5%. The proportion of individuals with
deletions in their toll like receptor 2 gene was 36.7%. However, the presence of this deletion within the TLR 2 gene was not statistically significantly associated with clinical outcome of the disease (P≥0.05). Our findings demonstrate a considerable low burden in filarial infection. However, there is clear evidence of ongoing transmission despite the 8 rounds of MDA. It is unlikely that the annual MDA would eliminate filarial transmission. The evidence of the presence of TLR 2 deletion genotypes in the 5’ untranslated region was found in individuals who reside in the endemic villages of Tanga region, North Eastern Tanzania, highlighting the potential for the susceptibility of bancroftian filariasis infection. These findings should inform the design of additional strategies to accelerate lymphatic filariasis control and elimination.
DECLARATION

I, Happyness Jeremia Mshana, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work and that it has neither been submitted nor being concurrently submitted to any other institution.

__________________  ________________
Happyness Jeremia Mshana                      Date
(MPhil. Candidate)

The above declaration is confirmed by:

__________________  ________________
Prof. Gerald Misinzo                               Date
(Supervisor)

__________________  ________________
Dr. Williams Makunde                               Date
(Co-Supervisor)
COPYRIGHT

No part of this dissertation may be reproduced, stored in any retrieval system or transmitted in any form or by any means without prior written permission of the author or Sokoine University of Agriculture in that behalf.
ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my supervisors Prof. Gerald Misinzo and Dr. Williams H. Makunde for the continuous support during this study and research, for their patience, motivation, enthusiasm, and immense knowledge. Their guidance helped me at all times of research and writing of this dissertation. In particular, I am grateful to Dr. Williams H Makunde for enlightening me the first glance of research. Besides my supervisors, I would like to thank Mr. Vito Baraka and Mr. Jubin Osei Mensah for their encouragement and insightful comments. My sincere thanks also goes to Prof. Achim Horeauf and Dr. Ken Pfarr, for offering me the summer internship opportunity in their laboratories that allowed me to work on diverse exciting projects. I thank my fellow laboratory mates at Tanga Medical Research Centre, Charles Guzo, Xavaeli Ngowi, Fidelis Mumburi, and Catherine Bakari for working together and for all the good moments we have had during the field work. Last but not the least, I would like to thank my family and friends for encouragement and spiritual support throughout my life, without forgetting the creator of the universe for the breath, energy and everything he is bestowing me.
DEDICATION

This work is dedicated to my father, the late Mr. Jeremia Mshana.
### TABLE OF CONTENTS

EXTENDED ABSTRACT ................................................................................................. ii
DECLARATION ........................................................................................................... iv
COPYRIGHT .............................................................................................................. v
ACKNOWLEDGEMENTS ............................................................................................ vi
DEDICATION ............................................................................................................. vii
TABLE OF CONTENTS ........................................................................................... viii
LIST OF ALL ABBREVIATIONS ............................................................................... x
LIST OF PAPERS ...................................................................................................... x

1.0 INTRODUCTION .................................................................................................. 1
  1.1 Background ........................................................................................................ 1
  1.2 Study Justification ............................................................................................ 4
  1.3 Study Objectives ............................................................................................... 5
    1.3.1 Main objective ............................................................................................... 5
    1.3.2 Specific objectives ........................................................................................ 5

2.0 GENERAL MATERIALS AND METHODS ....................................................... 6
  2.1 Study Design ..................................................................................................... 6

CHAPTER TWO ......................................................................................................... 7

Paper One ................................................................................................................. 7
Current epidemiological assessment of bancroftian filariasis in Tanga region,
North Eastern Tanzania ............................................................................................. 7
The association of Toll-like receptor 2 (TLR 2) genetic polymorphisms with susceptibility to clinical bancroftian filariasis infections ......................................................... 14

CHAPTER THREE ........................................................................................................... 24
3.0 GENERAL RESULTS, DISCUSSION ........................................................................ 24
3.1 General Results ....................................................................................................... 24
3.2 Discussion ............................................................................................................... 25

CHAPTER FOUR .......................................................................................................... 27
4.0 CONCLUSIONS AND RECOMMENDATIONS ..................................................... 27
4.1 Conclusions ........................................................................................................... 27
4.2 Recommendations ............................................................................................... 27
4.3 Limitation of the study ......................................................................................... 28

REFERENCES ............................................................................................................. 29
APPENDICES ............................................................................................................. 32
## LIST OF ALL ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADL</td>
<td>adenolymphangitis</td>
</tr>
<tr>
<td>ALB</td>
<td>albendazole</td>
</tr>
<tr>
<td>Ct</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DEC</td>
<td>diethylcarbamazine</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>L2</td>
<td>larvae stage two</td>
</tr>
<tr>
<td>L3</td>
<td>larvae stage three</td>
</tr>
<tr>
<td>LEC</td>
<td>lymphatic endothelial cells</td>
</tr>
<tr>
<td>MDA</td>
<td>mass drug administration</td>
</tr>
<tr>
<td>NF-Kb</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Prox 1</td>
<td>prospero homeobox 1</td>
</tr>
<tr>
<td>qPCR</td>
<td>real-time polymerase chain reaction</td>
</tr>
<tr>
<td>TLR</td>
<td>toll like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VEGFR-3</td>
<td>vascular endothelial growth factor receptor 3</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>

## LIST OF PAPERS

...

1.0 INTRODUCTION

1.1 Background

Lymphatic filariasis (LF), is a vector-borne parasitic disease of immense impact on the well-being of populations in endemic countries within the tropics. Clinically, the initial stage of the disease presents as an acute febrile condition and adenolymphangitis (ADL) (Shenoy et al., 2008). Later on it is characterized with progressive development of a scrotal swelling (hydrocele) and swelling of the legs (lymphoedema) which gradually lead to elephantoid type of oedema (Debrah et al., 2006). However, the pathogenesis of the disease is not yet clear to date, although there are several underlying aetiological factors implicated (Debrah et al., 2006). The disease has two clinical spectrum of presentation, one ending up with amicrofilaremic and pathologies due to down-regulation by cell-mediated responses and the other ends up with microfilaraemic presentation without pathologies (Pfarr et al., 2009).

Lymphatic filariasis is caused by mosquito that transmit the filarial parasites, Wuchereria bancrofti and Brugia malayi. It is estimated that 20% of population worldwide are at risk of acquiring the infection, and 120 million individuals have already been infected by lymphatic filariasis (Nuchprayoon et al., 2007). These infected individuals live in remote areas. A total of 39 countries in Africa and South of Sahara are said to be affected by lymphatic filariasis including Tanzania (WHO, 2013). In Tanzania alone it is estimated that more than 406m people are at risk of getting the infection and 6 million people are infected (Simonsen et al., 2013). In Tanga region studies have indicated that high microfilarial rate followed by hydrocele and elephantiasis.

Lymphatic filariasis is transmitted through third-stage filarial larvae (L3) of genus anopheline and culicine. The third-stage filarial larvae (L3) introduced into the host during a blood meal, penetrate into the bite wound which develops into adult’s worms
residing in the lymphatics (WHO, 2013). The female worms measure 80 to 100 mm in length and are 0.24 to 0.30 mm in diameter, while males measure about 40 mm by 0.1 mm. Adults form nests and produce microfilariae measuring 244 to 296 μm by 7.5 to 10 μm (Mwaniki et al., 2013). The worms have a tendency of forming the nest and exhibit nocturnal periodicity, except the South Pacific microfilariae which lack marked periodicity (Jemaneh et al., 1995). The microfilariae usually shift from lymphatic system into the blood. These microfilariae later are ingested by mosquitoes when feed on the blood meal and undergo development to form L2 and subsequently L3 larvae (Erickson et al., 2009). Complex immune response associated with host parasite interaction is thought to cause the varied clinical manifestations of lymphatic filariasis (Bockarie et al., 2010).

Figure 1: Life cycle of Wuchereria bancrofti

In the endemic communities, early infection (microfilaraemia) is usually acquired during childhood, and clinical disease observed later in life. It may cause temporary or permanent disability (Bandi et al., 2001). During chronic filarial infection, toxins produced by dead or adult worms trigger a massive inflammatory response.
During this inflammation, lymph angiogenesis plays a pivotal role in facilitating the resolution of oedema and mobilization of leukocytes and immune cells such as macrophages and dendritic cells (Bandi et al., 2001). Notably pro-inflammatory signals such as tumor necrosis factor (TNF), interleukin 6 (IL-6) and interleukin 1B (IL-1B) lead to production of vascular endothelial growth factor A (VEGF-A), vascular endothelial growth factor C (VEGFC), vascular endothelial growth factor receptor 3 (VEGFR-3), prospero homeobox 1 (Prox 1) and nuclear factor kappa B (NF-Kb) by a variety of cells indicating a role for these factors in lymphatic vessel formation during the inflammatory process (Debra et al., 2007).

Furthermore, the activation of NF-Kb pathway in lymphatic endothelial cells (LECs) up regulates Prox 1 and VEGFR-3, which renders lymphatic vessels more sensitive to VEGF-C and VEGF-D produced by leukocyte which act directly on the lymphatic and are believed to contribute to a change. Upregulation of these lymphangiogenic molecules, produced by endothelial cells that cause lymphatic dilation (Debra et al., 2007). The lymphatic vessels harboring the filarial parasite gradually become dilated with non-functional values, impaired contractility and abnormal drainage patterns. As a result of lymphoedema and chronic obstructive lesions in the lymphatic vessels due massive fluid accumulation causing irreversible damage to the lymphatic (Debra et al., 2007).

The clinical manifestation of lymphatic filariasis in the endemic population is influenced by the anatomic location of adult worms in a dilated nest within the lymphatic vessels, commonly in the extremities and male genitalia, the presence or absence of microfilaria and immunogenetics of the host and secondary bacterial infections (Babu et al., 2012). Those worms in the vessels they alter the structural anatomy of the vessels leading to lymphatic fluid accumulation which results into lymphoedema and irreversible obstructive lesions within the vessels and their walls (Pfarr et al., 2009).
The adult parasites are long lived and can live in for 6-8 years initiating the early pathogenesis of the lymphatic filariasis (Debra et al., 2007).

Lymphatic filariasis was identified as one of six infectious diseases that could potentially be eradicated. In 1997, the WHO passed a resolution to eliminate lymphatic filariasis as a public health problem by 2020 (WHO 2011). In accordance with current MDA programs, the mainstay chemotherapy against lymphatic filariasis and onchocerciasis are combinations of ivermectin (IVM), diethlycarbamazine (DEC) and albendazole (ALB) for LF and IVM for onchocerciasis (WHO 2011). The activity of these drugs is seen in their profound ability to kill MF as well as late embryonic stages inside the adult female worms. However, studies have shown that therapies have little effect on adult worms themselves (Kwarteng et al., 2016).

1.2 Study Justification

Lymphatic filariasis has shown to have two clinical spectra of presentation, one end with those amicrofilareamic and down-regulated through cell-mediated responses and having pathologies, whereas the other extreme are those microfilaraemics without pathologies (Pfarr et al., 2009). Other studies have indicated differential susceptibility to filarial infections within the families in populations and also polymorphisms in various genes have shown to be associated with clinical types of lymphatic filariasis. Such genes include VEGF A, mannose-binding lectin, human leukocyte antigen and chitotriosidase (Junpee et al., 2010). The actual cause of the disease is not yet known.

Toll-like receptors is a family of pattern recognition protein, which plays a major role in an innate immune system which in turn play a role in the adaptive immune system in recognising the materials derived from pathogens. Studies have indicated that mutations in TLR2 genes have effects in terms of susceptibility to infectious diseases e.g. leprosy,
tuberculosis and Lyme disease. However, a study in Thailand has indicated the association of TLR2 polymorphisms and asymptomatic bancroftian filariasis. Mutations of TLR2 gene have been shown to lower the transcription activity of TLR2 gene which results to the diminishing of TLR2 protein and hence the failure of the immune system to fully recognise the pathogens leading to high susceptibility to infection (Junpee et al., 2010). However, other studies have indicated that TLR2 polymorphisms do not correlate with lymphatic filariasis infection status or disease phenotype of lymphatic filariasis. Since there is limited data regarding TLR2 mutations, additional genotyping studies on TLR2 and other candidate genes involved in susceptibility to lymphatic filariasis are required to determine their contribution to the heterogeneous pattern of infection and filarial disease. Therefore, the aim of this study was to assess the role of deletion polymorphisms in TLR2 gene and their potential association to filarial infection. Exploring the role of the TLR2 polymorphisms is important to the understanding of the pathogenesis and protection against filarial infection.

1.3 Study Objectives

1.3.1 Main objective

To assess current clinical disease and infection status of lymphatic filariasis and its association with human genetic variability or linkage in Tanga region.

1.3.2 Specific objectives

- To determine the prevalence of the clinical disease, and
- To determine the association of TLR 2 mutations and bancroftian filariasis among Tanzanian cohorts in Tanga, Tanzania.
2.0 GENERAL MATERIALS AND METHODS

2.2 Study Area and Population

The study was conducted in Tanga region in North eastern Tanzania (05°04′S, 39°06′E). The region is characterised mainly by two rain seasons annually: the long rains from March to June and the less intensive short rains from November to December. The majority of inhabitants practice subsistence farming, fishing, and livestock keeping. The climate in Tanga region is warm and wet. In most cases, there is no big variation of temperature at the coast due to the influence of the Indian Ocean. Also, the region is characterised by high humidity, which often goes up to 100% maximum and ranges from 65 to 70% minimum. There are health facilities in most villages in the regions, and the majority of the population have access to a health facility within a distance of 6 km. Most of the houses are made of mud walls with their roofs thatched with dried coconut leaves. Lymphatic filariasis elimination program started in 2004 using MDA campaigns and advocacy with ivermectin and albendazole in all districts. The estimated population size of the area was 2,045,205 inhabitants according to 2012 national census survey.

2.1 Study Design

The study was a cross-sectional, descriptive study conducted in parallel with blood collection. The study participants were selected using convenient sampling, a nonprobability sampling technique where individuals were selected based on their convenient accessibility and proximity. The blood collected were further analysed using the real time PCR (qPCR) for the TLR 2 -196 to – 173 deletion detection and amplification. The extraction of DNA was done using the QIAmp® kit, according to manufacturers instructions. The real time PCR was done using the Rotor gene 6000 Real Time PCR system (Qiagen, Foster City, California 94 404, USA).
CHAPTER TWO

Paper One

Current epidemiological assessment of bancroftian filariasis in Tanga region, North Eastern Tanzania

Published in the Journal of Tropical Medicine
**The association of Toll-like receptor 2 (TLR 2) genetic polymorphisms with susceptibility to clinical bancroftian filariasis infections**

Happyness J. Mshana¹,² Vito Baraka¹,³ Gerald Misinzo²,⁴ and Williams H. Makunde¹

¹National Institute for Medical Research, Tanga Centre, P. o. Box 5004, Tanga, Tanzania.
²Department of Veterinary Microbiology, Parasitology and Biotechnology, College of Veterinary Medicine and Biomedical Sciences, Sokoine University of Agriculture, Morogoro, Tanzania.
³Global Health Institute, University of Antwerp, Antwerp, Belgium.
⁴SACIDS Africa Centre of Excellence for Infectious Disease of Humans and Animals in Southern and Eastern Africa, Morogoro, Tanzania.

**ABSTRACT**

**Background:** Lymphatic filariasis is a mosquito-borne parasitic disease. Lymphatic filariasis has been found to have an immense impact on the well being of populations in endemic countries within the tropics. The factors that underly severity of clinical outcome among individuals are not clearly understood, although deletions within the TLR2 gene have been implicated. This study was conducted to determine the association of deletions of nucleotides 196 to 173 (23 nucleotides) in the 5’ untranslated region of TLR2 gene with clinical outcome of bancroftian filariasis in endemic communities of Tanga region in Tanzania.

**Methods:** A total of 76 individuals were tested for TLR2 a 23 nucleotide deletion within the 5’ untranslated region using allele specific real time polymerase chain reaction (qPCR).
Results: Deletions of 23 nucleotides within the TLR2 gene was detected in 36.7 % of the participants. These participants had hydrocele, lymphoedema and circulating filarial antigen. However, the presence of this deletion within the TLR2 gene was not significantly associated with clinical outcome of the disease.

Conclusion: Toll like receptor 2 deletion polymorphisms genotypes occurrence among individuals infected with bancroftian filariasis disease highlights the potential for the susceptibility of bancroftian filariasis infection, although no association was found in the study. It is important that further genetic research be conducted to better understand the mechanism of infection transmission and heterogeneity of the disease.

INTRODUCTION

Lymphatic filariasis is the second leading cause of long-term disability globally due to lymphoedema, elephantiasis and hydrocele [1]. The long-term disability is triggered through overt hydrocoele, lymphoedema, elephantiasis, repeated orchitis, and adenolymphangitis. These acute and chronic pathologies impose a significant impediment to socioeconomic development and an extremely poor quality of life [2]. The pathogenesis of the disease is not yet clear to date. However, the disease has two clinical spectrum of presentation, amicrofilareamic form where the parasite is down-regulated through cell-mediated responses leading to pathologies and a microfilaraemics forms without pathologies [3].

The immune responses differ between individuals who present pathologies and those who do not. One plausible explanation for the different responses is that, single-nucleotide polymorphisms (SNPs) or deletions alter the expression of or the activity of immune factors such as the major histocompatibility (MHC) and toll like receptors [4]. On the
other hand, the presence of adult filarial worms in the lymphatic vessels and lymph nodes is also a factor known to trigger the pathology. Those worms in the vessels alter the structural anatomy of the vessels leading to massive accumulation of lymph fluid resulting into lymphoedema and irreversible obstructive lesions within the vessels and their walls [3].

Toll like receptors are pattern-recognition receptors (PRRs) that recognize evolutionarily conserved structures on the invading pathogens. Toll like receptors trigger a number of pro inflammatory and antimicrofilariae responses, through ligands binding for defence against the pathogens and promoting adaptive immunity responses [5]. Toll like receptor 4 is the signal transducing element of the lipopolysaccharide (LPS) receptor complex1 and is also involved in the signaling response to other exogenous stimuli. Toll like receptor 5 binds flagellin, a bacterial protein involved in motility and TLR 9 recognizes CpG, a repetitive sequence of unmethylated nucleic acids (cytosine and guanine).

Growing amounts of data suggest that single nucleotide polymorphisms (SNPs) on the various human toll like receptor proteins are associated with altered susceptibility to infection [6]. Toll like receptor 2, as a heterodimer with TLR1 or TLR6, two synonymous SNPs on TLR2 gene have been associated with asymptomatic bancroftian filariasis [7]. A-196 to -173 deletions polymorphisms in the 5’ untranslated region of TLR2 gene and two synonymous SNPs, 597C>T (rs3804099, Asn199ASN) and 1350C>T (rs3804100, Ser450Ser) in exon 3 are associated with susceptibility to asymptomatic bancroftian filariasis [8]. However other studies have indicated that TLR2 polymorphisms do not correlate with lymphatic filariasis infection status or disease phenotype of lymphatic filariasis [9]. Since there is limited data regarding TLR2 mutations in Tanzanian population, additional genotyping studies on TLR2 and other candidate genes involved in susceptibility to lymphatic filariasis will be required to determine their contribution to the
heterogeneous pattern of infection and filarial disease. Therefore, the aim of this study was to determine the role of deletion polymorphisms in TLR2 gene and their potential association to filarial infection. Exploring the role of the TLR2 polymorphisms is important to the understanding of the pathogenesis and protection against filarial infection.

MATERIALS AND METHODS

Study site

Tanga region lies between Latitude: -5° 04’ 8.15” S Longitude: 39° 05’ 55.50” E. It consists of different ethnic groups and large minority of Wazigua. The livestock species kept are primarily cattle, goats, sheep and donkey. The area has two rainy seasons: the short rainy season and the long rain season with annual precipitation between 500 and 1,000 mm. The study was carried out in villages of Tanga region located in North eastern Tanzania. The area which has known to be endemic for bancroftian filariasis. The districts included Tanga city, Pangani, Muheza and Mkinga.

Study design

The study involved the samples collected from previously cross sectional survey conducted in September 2016 in the study villages. Samples were collected from lymphatic filariasis (lymphoedema, hydrocele, CFA positives, 18-74 years old) patients residing in endemic communities of Tanga region.

Blood collection

Ten mls of EDTA monovette of blood were collected from each volunteer; plasma was separated and stored at -80 C. The blood pellets were mixed 1:1 with 8M Urea and transported to laboratory.
DNA extraction

Genomic DNA was extracted from peripheral blood preserved in an equal volume of 8M urea from using the QIAmp ® kit from Qiagen (Hilden, Germany) as per manufacturer’s instructions.

Plasmids preparation

Escherichia coli bacteria were grown on 15 mls of Lysogen Broth medium with appropriate temperature of 37° C overnight. Lysogen Broth medium allows bacteria that have been successful transformed to grow uninhibited. Overnight culture was then transferred to 1.5 mls Eppendorf tube and spin down the cell culture at high speed for 1 minute. 250 mls of lysis buffer solution were added and mixed gently by inverting the tube 6-8 times followed by 250 mls of neutralizing buffer solution. Bacteria precipitates were observed around the tube. Centrifugation were done at 13000 rpm for ten minutes then the supernatant was transferred to 1.5 mls Eppendorf tube and plasmid DNA pellets were resuspended by TE buffer.

Real time Polymerase Chain Reaction

The molecular detection of the of the TLR 2 -196 to -173 deletion polymorphism was performed using the RT-Polymerase chain reaction system (Rotor gene 6000 Real Time PCR system). The primers used were, TLR – 2 Forward: (5’- CGG AGG CAG CGA GAA A-3’) and TLR – 2 Reverse: (5’- CTG GGC CGT GCA AAG AAG-3’) and the TaqMan deletion probe (5’-AGC CAG GTG ACT GC- 3’) and wild type probe (5’- ACG CCG AGC AGC CG-3’) targeting 23 base pair fragment of the presence or absence of 23 nucleotides within the TLR 2 gene. The results of the assay were assessed by the Ct value.
Polymerase chain reaction parameters

The polymerase chain reaction (PCR) was carried as follows. The 20 µl reaction mixture contained 2 µl of 50 ng of template DNA, 10 µl of Quantinova master mix, 0.6 µl of forward and reverse primer respectively, 0.2 µl of wild type probe and deletion probe respectively, 6.6 µl of deionized water. The amplification was carried out using the Rotor gene 6000 Real Time PCR system. The amplification profile consisted of: 95° C for 2 minutes; followed by 45 cycles of 95 ° C for 5 seconds and 58° C for 30 seconds. The amplification of all DNA samples was repeated three times in order to see variability, if any in the amplification pattern.

Statistical analysis

FamHap software was used to carry the statistical analysis. The goodness of fit to Hardy–Weinberg Equilibrium (HWE) was performed using a chi-square (χ²) test. Determination of allele and genotype were determined by direct counting and then divided by the number of chromosomes to produce an allele frequency or by the number of subjects to produce the genotype frequency. Allele or genotype frequencies were compared between bancroftian filariasis patients, lymphoedema, hydrocele and normal controls to ascertain the association. P-values were considered significant at ≤0.05.

RESULTS

Deletions of 23 nucleotides within the TLR 2 gene was detected in 36.7 % participants. These participants had hydrocele, lymphoedema and circulating filarial antigen. However, the presence of this deletion within the TLR 2 gene was not significant associated with clinical outcome of the disease.
Table 1: The sex distribution of the patients genotyped

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>Sex (male, female)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circulating filarial antigen (CFA) patients</td>
<td>4</td>
<td>4M, 2F</td>
</tr>
<tr>
<td>Lymphedema patients</td>
<td>26</td>
<td>6M, 20F</td>
</tr>
<tr>
<td>Hydrocele patients</td>
<td>28</td>
<td>28M, 0F</td>
</tr>
<tr>
<td>Endemic normals</td>
<td>16</td>
<td>9M, 7F</td>
</tr>
</tbody>
</table>

Table 2: Genotype frequencies of TLR 2 -196 to –173 insertions/deletion in lymphatic filariasis patients

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>wt/wt OR</th>
<th>wt/del OR</th>
<th>del/del OR</th>
<th>P-Armitage value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphedema</td>
<td>17 1.5</td>
<td>0 0</td>
<td>9 0.818</td>
<td>0.622</td>
</tr>
<tr>
<td>Hydrocele</td>
<td>16 1</td>
<td>1 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bancroftian filariasis</td>
<td>4 1.556</td>
<td>0 0</td>
<td>2 0.944</td>
<td>0.952</td>
</tr>
<tr>
<td>Lymphoedema</td>
<td>17 1</td>
<td>0 9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bancroftian filariasis</td>
<td>4 1.059</td>
<td>0 0</td>
<td>2 0.643</td>
<td>0.658</td>
</tr>
<tr>
<td>Normal controls</td>
<td>9 0</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bancroftian filariasis</td>
<td>4 0.964</td>
<td>0 0</td>
<td>2 0.773</td>
<td>0.721</td>
</tr>
<tr>
<td>Hydrocele</td>
<td>16 1</td>
<td>1 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoedema</td>
<td>17 0.681</td>
<td>0 9</td>
<td>1.202</td>
<td>0.622</td>
</tr>
<tr>
<td>Hydrocele</td>
<td>16 1</td>
<td>1 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endemic normal</td>
<td>9 1.417</td>
<td>0 7</td>
<td>1.469</td>
<td>0.554</td>
</tr>
<tr>
<td>Lymphoedema</td>
<td>17 0</td>
<td>9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

This study has demonstrated the evidence of TLR 2 -196 to -173 polymorphism genotype in the 5’ untranslated region among individuals of Tanga region, North Eastern Tanzania. The study was carried 14 years after the implementation of the mass drug administration using the drug combination of ivermectin and albendazole with the aim of interrupting the bancroftian filariasis infection. During the course of the study, the cross sectional survey findings demonstrated a considerable low burden in filarial infection [1].

The TLR 2 haplotype -196 to -173 del/ +597C/+1350C have been reported to strongly associated with an increased risk of asymptomatic bancroftian filariasis in Thailand [8]. No previous reports of association have been reported in the area. A case control study conducted in Ghana revealed a strong association of TLR 2 -196 to -173 del with asymptomatic bancroftian filariasis. (Jubin et al., 2017 unpublished data). However, a similar study could not detect the association in individual tested in Tanga region. Other studies have demonstrated the role of polymorphisms in various host genes and
susceptibility and clinical manifestation of lymphatic filariasis such as Vascular Endothelial Growth Factors and Mannose-binding lectin [8]. However little is known regarding the pathogenesis of the disease and this calls for more research in the area for better understanding of the disease, considering the fact that there are potential mosquito vectors in the area, the risk of disease emergence is still high.

CONCLUSION

TLR 2 -196 to -173 deletion genotypes occurrence among individuals infected with bancroftian filariasis disease highlights the importance of further research for better understanding the mechanism of infection acquisition and heterogeneity of the disease.

REFERENCE


3.0 GENERAL RESULTS, DISCUSSION

3.1 General Results

A total of 472 individuals were examined during the community survey, among those 65.1% (307) were males while 34.9% (165) were females. Of those, 272 were recruited for the study of which 87.86% were males and 12.14% were females. The rest did not fulfil the inclusion criteria. The study population that was more frequently observed with high proportion was the age 32-73 with a rate of 24.2% followed by the 60-73 years with a rate of 33.1% and the majority of them were fishermen (62.5%).

Females were observed to have high proportion of lymphoedema than males at the age group of 32-45, 5.51% of the 272 individuals were CFA-positive and the majority of those were males (3.3%). Few of the confirmed CFA patients were females (2.21%). The proportion of males with hydrocele was higher (73.2%) than those with lymphoedema (15.8%). Conversely, the proportion of individuals with both lymphoedema and hydrocele was (5.51%).

A total of 76 individuals were genotyped, among those 61.8% (47) were males while 38.2% (29) were females. Deletions of 23 nucleotides within the TLR 2 gene was detected in 36.7% participants. These participants had hydrocele, lymphoedema and circulating filarial antigen. However, the presence of this deletion within the TLR 2 gene was not significant associated with clinical outcome of the disease.
3.2 Discussion

The results of this study suggest that during the era of lymphatic filariasis has shown a clear reduction of acute infection and morbidity rates compared to the baseline study especially in the older age group. However, there is still on-going low burden of filarial infection in some of the studied communities in the region and it is unlikely that, the annual single dose can eliminate the disease and therefore other strategies such as biannual administration of MDA can be applied to interrupt the transmission as observed in other studies.

It is vital to expand the frequency of drug administration to a maximum of three doses per year, expand the age range of the target population and improve health education to the community with the aim of increasing coverage within the targeted areas. Similarly conduct an anthropological study to find out why transmission continues although MDA is in place, tracking factors associated in that, such as the incentive to drug distributors, health system roles in the district, availability of drugs timely, financial resources, advocacy and training of distributors. The need for adequate financial and logistics resources is paramount to successfully achieve the targeted coverage and reach the end goal of the program. Adequate resources and infrastructural support should be available to ensure timely availability and supply of the MDA drugs to reach the implementation units in endemic communities.

In our study, hydrocele was observed to be the main public health problem and causing debilitation in males and similarly lymphoedema in females as observed elsewhere (Addis et al., 2007). It has also shown in our study these conditions are affecting the age group 32-45 years. Recent studies focus on molecular mechanism regulating blood and lymphatic vessels growth have shown that vascular endothelial growth factors control angiogenesis and lymphangiogenesis in humans (Pfarr et al., 2009), which is a process of
developing lymphangiactasia as a clinical disease. Similarly, expression of VEGF-A and VEGF-C have shown to be up-regulated by pro inflammatory cytokines affecting the lymphatic vessels in males (Pfarr et al., 2009). The TLR 2 haplotype -196 to -173 del/ +597C/+1350C have been reported to strongly associated with an increased risk of asymptomatic bancroftian filariasis in Thailand (Junpee et al., 2010). No previous reports of association have been reported in the area. A case control study conducted in Ghana revealed a strong association of TLR 2 -196 to -173 deletion with asymptomatic bancroftian filariasis (unpublished data). However, a similar study could not detect the association in individual tested in Tanga region. Currently, there is limited data on the heterogeneity of the disease. Since the pathogenesis and development of lymphoedema remain largely unknown, there is a need for future studies to explore the role of genetics in relation to clinical phenotypes to better understand the disease aetiology and optimize the control strategies.
CHAPTER FOUR

4.0 CONCLUSIONS AND RECOMMENDATIONS

4.1 Conclusions

This study has demonstrated a considerable low burden in filarial infection. However, there is clear evidence of ongoing transmission despite the 8 rounds of MDA using ivermectin and albendazole. It has been observed that lymphatic filariasis has different clinical manifestations. This study has demonstrated the evidence of the presence of TLR 2 -196 to -173 deletion genotypes in the 5’ untranslated region in individuals residing in Tanga region, North Eastern Tanzania. The presence of TLR 2 -196 to -173 deletion affects the gene expression at transcription level which in turn diminish the TLR 2 protein that plays an important role on the innate immune system and hence hinders the recognition of the pathogen, highlighting the potential for the susceptibility and presentation of bancroftian filariasis infection.

4.2 Recommendations

i. There is a need of investigating the genetic characteristics of individuals for better understanding the heterogeneity of the disease and mechanism of infection transmission. The approach will help to identify persons at risk before pathology is seen development of rapid screening test and hence, diseases monitoring and identification of new treatments.

ii. There is a need of investigating the molecular characteristics of the parasite in order to define their genetic and antigenic features that could play a greater role in designing an effective vaccine.
iii. It is unlikely that annual mass drug administration would interrupt filarial transmission; therefore, optimized strategies are needed to accelerate control and elimination of targets including mosquito vectors.

4.3 Limitation of the study

Due to limited resources only a subset of positive samples was subjected to qPCR analysis and this is considered to be limitation of this study.
REFERENCES


APPENDICES

Data Collection Form—Patient case report sheet-Tanzania (DFG-Genetics of Lymphedema and Hydrocele in Lymphatic Filariasis)


PATIENT ID NO:
Examination Form:

Patient Code NO:
Gender (1=Male, 2=Female)
Age (years)

Tribe: __________________________________________
Length of residence in years
Place of birth                  ______________________________
Village Name……………….
Occupation………………………………

Examined by:______________________________

QUESTIONS:

1. Do you have any health problems? (1=yes, 2=no)
2. Do you currently suffer from headaches? (1=yes, 2=no)
3. Do you currently suffer from chest pain? (1=yes, 2=no)
4. Do you currently suffer from stomach ache? (1=yes, 2=no)
5. Do you currently suffer from limbs/scrotal swelling? (1=yes, 2=no)
   If answer = 2, skip to next page
6. Does the swelling of limb(s)/scrotal consistently worse at one time? (1=yes, 2=no)
   If answer=2, skip to next page
7. Specify time(s) when swelling of limb(s)/scrotal is worse.
   (1=dry season, 2=long rainy season, 3=short rainy season)
8. Duration of the swelling (LE) [Years]--or since when......................
9. Previous history of trauma/injury on the limbs: (1=Yes; 2=No)
10. Duration of the scrotal swelling (Hydrocoele) in Years or since when..............
11. Other member of the family having LE? [1=Yes; 2=No] If yes association

Father .................
Mother................
Brother............... 
Sister................
Sons..................
Daughters……………  
Grand father…………  
Grand mother…………  
Cousin……………….  
Uncle…………………

**GLANDS (Lymphonodes) Swollen (ADL) (1=Yes, 2=No)**

| Right     | Axillar |          |          |
| Left      | Axilar  |          |          |
| Right     | Inguinal|          |          |
| Left      | Inguinal|          |          |
| Right     | Femoral |          |          |
| Left      | Femeral |          |          |

Funiculitis In Males

| Right Funiculitis |          |          |
| Left Funiculitis  |          |          |

**Urogenital Syndrome**

| Scrotal Skin Infected |          |          |
| Right Orchitis       |          |          |
| Left     Orchitis    |          |          |

Epididmytis

| Right Hydrocoele (stages) |          |          |
| Left Hydrocoele (stages)  |          |          |

**Lymphoedema Upper/Arms & Lower (Legs) (1=Yes; 2=No)**

| Right Leg (stages) |          |          |
| Left    Leg (stages) |          |          |
| Right Leg Elephantiasis |          |          |
| Left Leg Elephantiasis  |          |          |
Data Collection Form–Patient case report sheet-Tanzania

(DFG-Genetics of Lymphedema and Hydrocele in Lymphatic Filariasis)


Laboratory Examination Sheet
Village Name: ________________________________  
Patient Code N0………  
Blood specimen collected? [1=Yes; 2=No] ………………………  
Volume collected in mLs  
Blood specimen collected by________________________date______________  
Time collected in Hrs (24Hrs)  
Trop Bio test Results: .antigens /units  
Microscopy for mff through filtration: Mff /mL……………….  
Time microscopy conducted__________________________Hrs.  
Time blood collected in (24Hrs):  
Volume of blood collected in mLs  
Collecting method ___________________________ (syringe, vacutainer)  
Time serum was separated in (24hrs)  
Volume of sera separated for each subject in mLs  
Time sera frozen at - 70o C in (24hrs)