

Molecular Detection of *Wuchereria bancrofti* in Human Blood and Mosquitoes from Selected Endemic Areas in Egypt

Thesis

Submitted in partial fulfillment of the
M.D. degree in Medical Parasitology

By

Iman Raafat Mohamed Riad

M.B.B.Ch., M.Sc.

Medical Parasitology Department
Faculty of Medicine – Cairo University

Supervised by

Prof. Dr. Ayman Abdel-Moamen El Badry

Professor of Medical Parasitology
Faculty of Medicine – Cairo University

Assistant Prof. Dr. Eman Yassien Shoeib

Assistant Professor of Medical Parasitology
Faculty of Medicine – Cairo University

Dr. Samar Sayed Attia

Lecturer of Medical Parasitology
Faculty of Medicine – Cairo University

In collaboration with

Dr. José Miguel Rubio

Head of Malaria & Emerging Parasitic Diseases Lab
National Microbiology Center – Carlos III Health Institute
Madrid – Spain

Faculty of Medicine
Cairo University
2015

Abstract

Lymphatic filariasis is a vector-borne health problem, which have a profound impact on patient's lives and can lead to permanent disability. *Wuchereria bancrofti* (*W. bancrofti*) is the major cause of filariasis worldwide and is focally endemic in Egypt. Diagnosis of filarial infection using traditional morphologic and immunological criteria can be challenging and lead to misdiagnosis. The aim of the present cross-sectional study was the molecular detection of *W. bancrofti* in human blood and mosquitoes from selected endemic areas in Egypt. Blood samples were collected from 300 individuals residing in filariasis endemic areas, and were subjected to ELISA for the detection of *W. bancrofti* antigens and semi-nested PCR targeting *W. bancrofti* repeated DNA sequences. Mosquito pools from same endemic areas were collected, sorted, and subjected to semi-nested PCR. Additionally, a group of positive PCR products were subjected to DNA sequencing and phylogenetic analysis. Results revealed that out of the 300 collected blood samples; 7 samples were positive by ELISA (2.3%), 45 samples were positive by semi-nested PCR (15%) and 3 samples were positive by both tests (1%). All the collected mosquito pools were negative. Sequences analysis confirmed semi-nested PCR results; identifying only *W. bancrofti* species. Sequence alignment and phylogenetic analysis indicated genetically distinct clusters of *W. bancrofti* among the study population.

Key Words: *Wuchereria bancrofti*, phylogenetic analysis, semi-nested PCR, repeated DNA sequence, ELISA, blood samples, mosquitoes

Acknowledgments

First and foremost, I would like to thank ALLAH Who paved the way and only by His will everything can be achieved.

Words are not enough to express how thankful I am to Professor **Dr. Ayman Abdel-Moamen El Badry** for being my mentor and guide in the field of molecular parasitology, and a generous source of knowledge and patience. His positive energy and immense support during all stages of the study made it possible to overcome problems and to carry this work forward.

I owe my deepest gratitude to Assistant Professor **Dr. Eman Yassien Shoeib** for her friendly caring and relentless devotion in all the time of research and writing. Her scientific advice, insightful discussions and suggestions were particularly valuable for this work to be done.

I am highly obliged and grateful to **Dr. Samar Sayed Attia**, whose guidance and continuous support throughout my academic career along with her truthful, illuminating views helped me to develop my background in research and to complete the present work.

I am greatly indebted to Professor **Dr. José Miguel Rubio** for giving me the opportunity to work at the Malaria & Emerging Parasitic Diseases Lab (MAPELab), Carlos III Health Institute, for introducing me to the sequencing analysis and phylogenetics, and lending me his expert views and precious time.

I am heartily thankful to the amazing staff of the **MAPELab**, especially **Thuy-Huong Ta Tang**, a wonderful and generous friend who has been giving me advice and opinions on lab related issues.

My sincere thanks to **Yusuf Edmardash**, Department of Entomology, Faculty of Science, Cairo University for his precious contribution in the identification of the collected mosquitoes and other unidentified flies specimens.

I greatly appreciate and wish to thank all the staff of the **Malaria, Filariasis and Leishmaniasis Control Department, Ministry of Health and Population** for their kind permission, guidance and assistance in the collection of the material for the study.

Finally, in full gratitude I would like to acknowledge Professor **Dr. Mona Mahmoud Aly** and the wonderful professors and colleagues at the **Parasitology Department** who encouraged, inspired, supported and helped the pursuit of a high education degree.

Table of Contents

List of Abbreviations	page iii
List of Figures	page v
List of Tables	page viii
1. Introduction.....	page 1
2. Aim of Work.....	page 3
3. Literature Review	
3.1 Historical Note.....	page 4
3.2 Parasite Classification and Life Cycle.....	page 6
3.3 Epidemiology.....	page 10
3.4 Infection and Clinical Features.....	page 21
3.5 Control and Elimination Program.....	page 28
3.6 Diagnosis.....	page 30
4. Material and Methods	
4.1 Study Setting and Sampling	page 52
4.1.1Blood Samples	page 52
4.1.2Mosquito Samples	page 56
4.2 Laboratory Tests and Procedures	page 57
4.2.1Blood Samples	page 57
4.2.2Mosquito Samples	page 78
4.3 Data Analysis	page 84
5. Results	page 85
6. Discussion.....	page 108
7. Conclusions	page 129
8. Recommendations	page 130

9. Summary	page 131
10. References.....	page 133
11. Appendix	
1	الملخص العربى

List of Abbreviations

Ab	Antibody
ADLA	Acute Dermatolymphangioadenitis
AFL	Acute Filarial Lymphangitis
Ag	Antigen
AM	Ante Meridiem
AT	Adenine - Thymine
B.	<i>Brugia</i>
bp	Base pair(s)
CFA	Circulating Filarial Antigens
Cx.	<i>Culex</i>
D.	<i>Dirofilaria</i>
DALYs	Disability-Adjusted Life Years
ddH₂O	Double Distilled Water
DEC	Diethylcarbamazine
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
ddNTPs	Dideoxynucleotide Triphosphates
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunsorbent Assay
FDS	Filarial Dance Sign
fg	Femtogram
FNAC	Fine Needle Aspiration Cytology
GPELF	Global Programme to Eliminate Lymphatic Filariasis
HRPO	Horseradish Peroxidase
ICT	Immunochromatographic Test
Ig	Immunoglobulin
Kb	Kilo base pairs
kDa	Kilo Dalton
L1	First -stage larvae
L2	Second-stage larvae
L3	Third-stage larvae
L4	Fourth-stage larvae
LAMP	Loop-Mediated Isothermal Amplification
LF	Lymphatic Filariasis
M.	<i>Mansonella</i>
mAb	Monoclonal Antibody
Mb	Mega base pairs

MDA	Mass Drug Administration
Mf	Microfilariae
MgCl₂	Magnesium Chloride
MOHP	Ministry of Health and Population
NCBI-BLAST	National Center for Biotechnology Information – Basic Local Alignment Search Tool
NTD	Neglected Tropical Diseases
<i>O.</i>	<i>Onchocerca</i>
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pg	Picogram
PM	Post Meridiem
QBC	Quantitative Buffy Coat
q-PCR	Real Time - Polymerase Chain Reaction
rDNA	Ribosomal – DNA
spp.	Species
TBE	Tris Borate EDTA
TMB	3,3',5,5' - Tetramethylbenzidine
TPE	Tropical Pulmonary Eosinophilia
UV	Ultraviolet
<i>W.</i>	<i>Wuchereria</i>
WHA	World Health Assembly
WHO	World Health Organization
YLDs	Years Lived with Disability
YLLs	Years of Life Lost

List of Figures

Figure	Page
Figure 3.1: Depiction of elephantiasis in ancient civilizations	5
Figure 3.2: Diagram of <i>W. bancrofti</i>	7
Figure 3.3: Life cycle of <i>W. bancrofti</i>	9
Figure 3.4: Geographic distribution of LF	11
Figure 3.5: Map of Egypt indicating the governorates where LF is endemic	12
Figure 3.6: Diagrammatic representation of the gonotrophic cycle of a female mosquito	15
Figure 3.7: Development of life stages of LF parasite in a mosquito in relation to the gonotrophic cycle	19
Figure 3.8: <i>W. bancrofti</i> larvae in mosquito	19
Figure 3.9: Acute manifestations of LF	24
Figure 3.10: Lymphoedema & elephantiasis of lower limb	26
Figure 3.11: LF chyluria showing milky urine with blood	26
Figure 3.12: <i>W. bancrofti</i> Mf in a hematoxylin- stained thick blood film	32
Figure 3.13: Adult <i>W. bancrofti</i> in tissue section	34
Figure 3.14: Adult <i>W. bancrofti</i> in FNAC	35
Figure 3.15: Alere Filariasis Test Strip and BinaxNOW Filariasis card test performed with the same blood sample	42
Figure 3.16: Advantages and disadvantages of conventional PCR, multiplex PCR (mPCR), real-time PCR (qPCR) and LAMP	49
Figure 4.1: Google earth map showing areas of sample collection in Qualioubiya governorate	53
Figure 4.2: Google earth map showing areas of sample collection in Menoufiya and Giza governorates	54
Figure 4.3: Mosquito trap	56
Figure 4.4: Work scheme of blood samples	57
Figure 4.5: TropBio ELISA kit for detecting and quantifying <i>W. bancrofti</i> antigens	58

Figure 4.6:	ELISA configuration (Ag detection) for <i>W. bancrofti</i>	59
Figure 4.7:	Sample preparation for <i>W. bancrofti</i> TropBio ELISA test	61
Figure 4.8:	ELISA plate template	61
Figure 4.9:	Typical standard Ag curve	63
Figure 4.10:	The ELISA reader with the plate inside	63
Figure 4.11:	QIAamp DNA Blood Mini Kit – Qiagen – Germany	64
Figure 4.12:	DNA extraction flowchart	66
Figure 4.13:	Biometra T Personal Thermocycler	71
Figure 4.14:	Gel electrophoresis apparatus and power supply	73
Figure 4.15:	Work scheme of mosquito samples	78
Figure 4.16:	DNeasy Blood & Tissue Kit – Qiagen – Germany	79
Figure 4.17:	Sonicator used for homogenization of samples	81
Figure 5.1:	Residence of the study group	86
Figure 5.2:	Distribution of the study group according to LF condition	87
Figure 5.3:	Distribution of LF cases in study group by LF duration	88
Figure 5.4:	Clinical manifestations of the LF cases and the clinically suspected cases	89
Figure 5.5:	ELISA plate for <i>W. bancrofti</i> Ag detection	92
Figure 5.6:	Distribution of Ag positive cases in titre groups	92
Figure 5.7:	Distribution of ELISA positive results according to sample collection time	93
Figure 5.8:	Detection of <i>W. bancrofti</i> DNA using semi-nested PCR on ethidium bromide stained agarose gel (A)	94
Figure 5.9:	Detection of <i>W. bancrofti</i> DNA using semi-nested PCR on ethidium bromide stained agarose gel (B)	94
Figure 5.10:	Distribution of semi-nested PCR positive results according to sample collection time	95
Figure 5.11:	Total positive cases of <i>W. bancrofti</i> infection using ELISA and semi-nested PCR	97
Figure 5.12:	Distribution of <i>W. bancrofti</i> positive cases in relation to gender	98

Figure 5.13:	Distribution of <i>W. bancrofti</i> positive cases into age groups	99
Figure 5.14:	Distribution of <i>W. bancrofti</i> positive cases in relation to residence	100
Figure 5.15:	Distribution of <i>W. bancrofti</i> positive cases according to LF status	101
Figure 5.16:	Distribution of the positive cases in relation to the LF status of the household members	102
Figure 5.17:	Distribution of <i>W. bancrofti</i> positive cases in relation to MDA intake	103
Figure 5.18:	Multiple sequence alignment of the sequenced DNA and the reference <i>W. bancrofti</i> sequences on NCBI-BLAST	105
Figure 5.19:	Neighbour-Joining analysis of the phylogenetic relationship between the obtained <i>W. bancrofti</i> sequences and the reference sequences	106

List of Tables

Table	Page
Table 3.1: Milestones in filariasis history	5
Table 3.2: Various mosquito vectors of filariae causing human LF based on articles by WHO	13
Table 4.1: Allocation of samples to titre groups	64
Table 5.1: Gender distribution in the study group	85
Table 5.2: Age distribution in the study group	85
Table 5.3: Residence of the study group	86
Table 5.4: Distribution of the study group according to LF status	87
Table 5.5: LF duration in asymptomatic and chronic cases	88
Table 5.6: Clinical manifestations of the LF cases and the suspected cases	89
Table 5.7: Distribution of the study group according to the LF status of household members	90
Table 5.8: Distribution of the study group according to MDA intake	90
Table 5.9: Distribution of the study group according to sample collection time	91
Table 5.10: Results of <i>W. bancrofti</i> Ag detection ELISA test	92
Table 5.11: Distribution of ELISA positive results according to sample collection time	93
Table 5.12: Results of semi-nested PCR targeting <i>W. bancrofti</i> repetitive region	94
Table 5.13: Distribution of semi-nested PCR positive results according to sample collection time	95
Table 5.14: Relation between ELISA and semi-nested PCR results	96
Table 5.15: Distribution of <i>W. bancrofti</i> positive cases in relation to gender	98

Table 5.16:	Distribution of <i>W. bancrofti</i> positive cases in relation to age	99
Table 5.17:	Distribution of <i>W. bancrofti</i> positive cases in relation to residence	100
Table 5.18:	Distribution of <i>W. bancrofti</i> positive cases in relation to LF status	101
Table 5.19:	Distribution of the positive cases in relation to the LF status of the household members	102
Table 5.20:	Distribution of <i>W. bancrofti</i> positive cases in relation to MDA intake	103
Table 5.21:	The identified <i>Culex</i> Species and other dipterous flies	107

1.INTRODUCTION

1. INTRODUCTION

Lymphatic filariasis (LF) is a major vector-borne public health problem affecting more than 120 million people in over 80 endemic developing countries. *Wuchereria bancrofti* (*W. bancrofti*) which is responsible for 90% of cases throughout the tropics and in some subtropical areas worldwide is focally endemic in Egypt (**Melrose, 2002 & Foo *et al.*, 2011**).

The disease has been identified by World Health Organization (WHO) as the second leading cause of permanent and long term disability. In addition to medical problems, there are severe social and psychological consequences especially in those who suffer from elephantiasis or hydrocoele. LF also has a vast economic impact upon endemic communities resulting from the direct costs incurred in medical and surgical treatment, in addition to indirect costs from reduced work capacity and labour loss (**Melrose, 2002**).

To combat this disease, the WHO has launched a “Global Program to Eliminate Lymphatic Filariasis” (GPELF), aiming to eliminate LF by the year 2020 in all the endemic countries, using mass drug administration (MDA) to interrupt the disease transmission. Egypt was one of the first countries to implement a national program to eliminate LF based on WHO’s strategy of repeated rounds of annual treatment in the form of albendazole with diethylcarbamazine (DEC). Subsequently, transmission was shown to be markedly reduced among villages that prior to MDA exhibited some of the highest rates of LF (**Hotez *et al.*, 2012 & Upadhyayula *et al.*, 2012**).

The exponential growth of LF elimination program has highlighted the need for sensitive tools that can be used to monitor progress towards programmatic

endpoints. As well as, to conduct surveillance, rapid and early detection of cases that can be often challenging for several reasons (**Lammie *et al.*, 2004**).

For instance, LF is characterized by a wide range of clinical presentations. One group of individuals in the endemic community shows no clinical manifestations or microfilariae (Mf). This includes individuals who have not been sufficiently exposed to be infected, individuals with prepatent infection or adult worm infection without Mf, and individuals who have cleared the infection. Another group of individuals in the endemic community shows Mf in their blood but no obvious clinical manifestations. Some of these may remain microfilaraemic and asymptomatic for years or even for the rest of their lives, while the rest may become symptomatic (**Simonsen, 2009**).

As regards the laboratory diagnosis of LF, the night time blood collection for Mf detection and the low intensity of parasites have created a problem which have led to the development of alternative methods for diagnosis. Immunological assays are reasonably sensitive and more specific than the earlier tests. The detection of specific circulating filarial antigens (CFA) are extensively used by the WHO for field diagnosis of LF, however the cost and inconsistent availability remain drawbacks to antigen detection tests (**Nuchprayoon, 2009**).

Recent studies have shown that filarial DNA could be detected in human blood and in mosquito blood meals by polymerase chain reaction (PCR) based assays, which offer the possibility of improved sensitivity and specificity. Moreover, recent advances in molecular biological technology are giving parasitologists new insights into the structure and function of the filarial genome (**Kanjanavas *et al.*, 2005** and **Liu & Austin, 2013**).