MOLECULAR DETECTION OF *WOLBACHIA* AND FILARIAL NEMATODE IN ANTHROPOPHILIC MOSQUITOES IN THE GAMBIA

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NIGERIA

APRIL, 2018

DECLARATION

I declare that the work in this Thesis entitled "Molecular detection of *Wolbachia* and filarial Nematode in anthropophilic mosquitoes in The Gambia" has been carried out by me in the Department of Biochemistry, Faculty of Life Science ABU, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any other Institution.

Sankung SANNEH _____ _____

(NAME) (SIGNATURE) (DATE)

CERTIFICATION

This thesis entitled MOLECULAR DETECTION OF WOLBACHIA AND FILARIAL NEMATODE IN ANTHROPOPHILIC MOSQUITOES IN THE GAMBIA by SANKUNG SANNEH meets the regulations governing the award of the degree of Master (M.Sc.) of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

This thesis is dedicated to my late brother ALAGIE SANNEH, whose main desire was to see me go through my educational pursuit. God did not grant him the chance to fulfill his desire as he died at early age.

May Allah grant him and those who went before him Jannatul Firdawsi.

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ABSTRACT

This study was embarked upon to provide a baseline information on the incidence of *Wolbachia* in anthropophilic mosquitoes as well as to provide information on active transmission of lymphatic filariasis in The Gambia using polymerase chain reaction. The study was conducted in 48 settlement (8 from each division). A total of 2003 mosquitoes pooled into 120 clusters from the 48 settlements in the country were classified by microscopic technique into genera. The DNA of the mosquitoes by cluster were examined using polymerase chain reaction and gel electrophoresis for the presence of *Wolbachia* and filarial nematode using 16SrDNA for *wolbachia* and SspI for *Wuchereria bancrofti* and HhaI for *Brugia malayi*. The positive samples for *Wolbachia* were further differentiated into supergroup of *wolbachia* using the primer wsp136F/wsp691R for supergroup A and primer wsp81F/wsp522R for supergroup B. The level of association between *wolbachia* and the genera of anthropophilic mosquitoes in the Gambia was also determined. Microscopic results shows 64.9% *Anopheles,* 32.0% *Culex, 3.0% Aedes.* There were no *Mansonia* mosquito genera detected. Using 16srDNA primers, an incidence of 34.17% of *Wolbachia* in the mosquito were obtained which varied among the three genera, highest being among Anopheles. Only *Wolbachia* supergroup B was identified in Culex and Ades. The level of association was found to be significant ($P \leq 0.05$) only in Culex anthropophilic mosquitoes. For the 120 cluster of mosquitoes screened for the presence of *Wuchereria bancrofti* and *Brugia malayi,* non of the clusters were positive.

TABLE OF CONTENTS

DECLARATION iii

CERTIFICATION iv

DEDICATION v

ACKNOWLEDGEMENT vi

ABSTRACT viii

Table of Contents ix

LIST OF TABLES xiv

LIST OF FIGURES xv

LIST OF PLATES xvi

LIST OF APPENDICES xvii

ABBREVIATION xviii

1.0 INTRODUCTION 1

1.1 BACKGROUND 1

1.2 Statement of Research Problem 8

1.3 JUSTIFICATION OF STUDY 9

1.4 AIM 10

1.5 SPECIFIC OBJECTIVES 10

1.6 HYPOTHESIS 11

2.0 LITERATURE REVIEW 12

2.1 Filariasis 12

2.2 Signs and symptoms of lymphatic filariasis 12

2.2.1 Physical symptoms 12

2.2.2 Psychological symptoms 13

2.3 Social impact OF LYMPHATIC FILARIASIS 13

2.4 Epidemiology of lymphatic filariasis 14

2.5 Classification and Morphology of lymphatic filarial worm 16

2.5.1 *Wuchereria bancrofti* (*W. bancrofti*) 16

2.5.2 *Brugia malayi (B. malayi*) 18

2.5.3 Brugia timori (B. timori) 20

2.6 lymphatic filariasis vectors 22

2.6.1 The Genus: *Anopheles* 23

2.6.2 The Genus *Culex* 26

2.6.3 The genus: *Aedes* 28

2.6.4 The genus *Mansonia* 31

2.6.5 LIFE CYCLE OF MOSQUITO 33

2.7 Diagnosis of Lymphatic filariasis 36

2.7.1 Microscopical detection method 36

2.7.2 Serological technique (Antibody detection) 37

2.7.3 Molecular detection technique 37

2.8 Management and control of lymphatic filariasis 39

2.8.1 Treatment 39

2.8.2: Prevention and control of Lymphatic filariasis 41

2.9 *WOLBACHIA* ENDOSYMBIONT 42

2.10 *Wolbachia* and its arthropod host 45

2.11 *Wolbachia* and its nematode host 45

2.12 Transmission of *wolbachia* 46

2.13 Detection of *Wolbachia* in Mosquitoes 46

2.13.1 Near infrared spectroscopy 46

2.13.2 Polymerase Chain reachion Technique 47

3.0 MATERIALS AND METHODS 49

3.1 CHEMICALS AND REAGENTS 49

3.1.1 EQUIPTMENT 50

3.2 STUDY AREA 51

3.3 SAMPLE SIZE 53

3.4 LIMITATIONS 53

3.5 SAMPLING TECHNIQUES 53

3.6 STUDY UNITS 54

3.7 SAMPLING SITES 54

3.8 collection OF MOSQUITO SAMPLES 55

3.8.1 Collection of mosquitoes using adaptation of the G' rumba container 55

3.9 IDENTIFICATION OF THE MOSQUITOES 57

3.10 IDENTIFICATION OF *WOLBACHIA* AND MICROFILARIAE 58

3.10.1 DNA extraction 58

3.10.2 DNA quantification using Nanodrop Spectrophotometer 59

3.10.3 Identification of Wolbachia DNA using 16SrDNA and wsp gene 59

3.10.4 Identification of Microfilariae Species 62

3.10.5 Gel electrophoresis 64

3.11 STATISTICAL ANALYSIS 65

4.0 RESULTS 66

4.1 Purity assessment of the extracted DNA 66

4.2 Distribution of Anthropophilic Mosquito Genera In The Gambia. 66

4.3 Sexual Dimorphism of Anthropophilic Mosquito Genera 69

4.5 *Wolbachia* – Mosquito Association 77

4.6 Screening for the presence of *W.Bancrofti* and *M. Malayi* DNA in Anthropophilic Mosquitoes. 79

5.0 DISCUSSION 81

6.0 SUMMARY, CONCLUSION and RECOMMENDATION 84

6 1 Summary 84

6.2 Conclusion 85

6.3 Recommendation 85

6.4 Contribution To Knowledge 86

LIST OF TABLES

Table 2.1 Supergroup of *Wolbachia*, their host and relationship with host……..…44

Table 2.2 Sets of primers for identification of *Wolbachia* supergroup A and B……...…48

Table 3.1 Sampling sites in each division…….53

Table 4.1 Population of anthropophilic mosquitoes by genera in different division in the Gambia……...66

Table 4.2 Sexual dimorphism of anthropophilic mosquitoes in different divisions in the Gambia……70

Table 4.3 Pooled sample of *Wolbachia* incidence in anthropophilic mosquitoes genera in the Gambia……...75

Table 4.4 Distribution of *Wolbachia* supergroup among anthropophilic mosquitoes in the Gambia……...77

Table 4.5 Bivariate fit of anthropophilic mosquito genera and *Wolbachia* in the Gambia…...79

LIST OF FIGURES

Figure 2.1 Global distribution of Lymphatic filariasis highlighting the types…….15

Figure 3.1 Map of the Gambia indicating the six divisions……...……51

Figure 4.2 Pooled sample of anthropophilic mosquitoes in different division in the Gambia…..68

LIST OF PLATES

Plate 1.1 : Elephantiasis of the leg……...3

Plate 1.2 : Elephantiasis of the scrotum ……..3

Plate 2.1 Struture of *Wuchereria bancrofti*……...…17

Plate 2.2 Structure of *Brugia malayi*……..19

Plate 2.3 Structure of *Brugia timori*……..……21

Plate 2.4 *Anopheles* mosquito……..25

Plate 2.5 *Culex* mosquito……..……27

Plate 2.6 *Aedes* mosquito……..30

Plate 2.7 *Mansonia* mosquito ……..32

Plate 2.8 The life cycle of mosquito……..…35

Plate 2.9 *Wolbachia* endosymbiont…….…..43

Plate 2.10 G' rumba……..….55

Plate 2.10.1 G' rumba trap modified……..…55

Plate I: PCR amplification of the 16SrDNA (438bp) of *Wolbachia* in anthropophilic mosquito……..…….72

Plate II: PCR amplification of 16SrDNA (438bp) of *Wolbachia* in anthropophilic mosquito ……..…….73

Plate III : PCR amplification of wsp (442bp) for *Wolbachia* supergroup B in anthropophilic mosquito……..……75

Plate 4.3 PCR amplification of sspI (188bp) and hhaI (320bp) of *W. bancrofti* and *B. malayi* respectively in anthropophilic mosquito …….....80

LIST OF APPENDICES

Appendix I Number of *Anophles* , *culex, Aedes* and *Mansonia* per division…….……..98

Appendix II Number of Clusters in each division per genera…….99

Appendix III PCR assay for *Wolbachia* detection in anthropophilic mosquitoes in the Gambia…….….100

Appendix X PCR assay results for *Wolbachia* detection in anthropophilic mosquitoes in the Gambia……..101

Appendix XI PCR results for *Wolbachia* supergroup B……...…… 102

ABBREVIATION

B.malayi : Brugia malayi

Bp : Base pairs

B.timori : Brugia timori

DNA : Deoxyribonuclei acid

DEC : Diethylcarbamazine

dNTP : Deoxynucleotide Triphosphate

CRD : Central River Division

ELIZA : Enzyme- Linked immunosorbent Assay

EDTA : Ethylene-Diamine-tetra-Acetic acid

LRD : Lower River division

MDA : Mass drug administration

mRNA :Messenger Ribonucleic acid

RNA : Ribonucleic acid

NTD : Neglected tropical disease

NBD : North bank division

PCR : Polymerase chain reaction

PCR-RFLP : Polymerase chain reaction Restriction frangment length polymorphism

SMD : Saint Marys Division

16srDNA : Small subunit ribosomal deoxyribonucleic acid

TAE : Tris- Acetate- Ethylene-Diamine-tetra-Acetic acid

Taq DNA : Thermus Aquaticus Deoxyribonuclei Acid

URD : Upper river division

WD : Western Division

WHO: World Health Organisation

Wolb : *Wolbachia*

WSP : *Wolbachia* surface protein

W. bancrofti : Wuchereria bancfroti

CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

According to WHO (2013), lymphatic filariasis is one of the World's leading causes of permanent and long-term disability. Disfigurement can be in the form of enlargement of the entire leg (Plate 1.1) or arm, breasts and genitals (Plate 1.2). This happens when the nematode attacks the lymph vessels that maintain the delicate balance between the tissue and the blood.

Lymphatic filariasis, commonly known as elephantiasis is a disease that weakens and disfigures infected person. The disease is caused by thread- like filarial worms (microfilariae*): Wuchereria bancrofti, Brugia malayi* or *Brugia timori* (Lambert, 2005), that weaken and disfigure a victim of the disease. The adult worms which could measure up to 4 –12 cm long, house and mature as adults in the body's lymph nodes and lymph vessels for a period of 4 to 8 years (Bockarie *et a*l., 2009; Pfarr *et al*., 2009)

Of the three species that causes lymphatic filariasis, *Wucheria bancrofti* is most widely spread and is responsible for more than 90% of infection (Michael *et al.,* 1996). Microfilariae of *W. bancrofti* exhibit nocturnal periodicity (Nwoke *et al.,* 2010); during the day, they are found in the deep veins and during the night between 10 pm and 4 am, they migrate to the peripheral circulation. This behavior gives them the chances of being picked up by the night biting vectors during their blood meals (Nwoke *et al*., 2010)

The specie of mosquitoes capable of transmitting these filarial nematodes are many and varied. In most urban and semi urban areas, *W. bancrofti* is vectored mainly by *Culex* mosquito while in the rural areas, transmission from one person to another is mainly by anopheles mosquito (Singh

et al., 2013). Its main vectors in The Gambia are *Anopheles gambiae* in the rural areas while in the urban areas transmission is by *Culex quenquifasciatus.* In the rural areas, transmission of lymphatic filariasis is by *Anopheles gambiae*,while in the urban area , transmission is by *Culex quenquifasciatus.*



Plate 1.1 Elephantiasis of the leg (WHO, 2013



Plate 1.2: Elephantiasis of the scrotum (WHO, 2013)

The worms have an active reproductive period of 4-6 years, producing millions of small immature larvae, microfilariae, which circulate in the peripheral blood (WHO, 2013). The stages of development of the filarial worm are; egg, larva and adult worm. The adult worms between the age of 5-10 years mate and the female worm produces thousands of microfilariae which migrates into the bloodstream. A mosquito vector during blood meal can bite the infected human host and ingest the microfilariae. Inside the mosquito, microfilariae develop in about 12 days into larvae (Lambert, 2005) .These larvae are infectious to humans and if a mosquito carrying them bites a person, the larvae are injected into the bloodstream. Some larvae will migrate to the lymphatic system and develop into mature worms.

Infections usually occur during childhood but the observable symptoms occur during adulthood. The symptoms include pain , swollen limbs and bad smelling lesions in the skin, enlargement of the arms, legs, breasts, or vulva, difficulty in walking and mobility; damage to the lymphatic system and kidneys. In some cases the manifestations includes hydrocele and lymphedema which are as a result of accumulating, worm-induced damage in the lymphatic system (Lambert, 2005). Hydrocele is an enlargement of the scrotum in males, caused by accumulation of serous fluid inside the scrotal sac, around the testicles (Plate 1.2) while lypmphoedema is a swelling of extremities, breast or vulva, caused by accumulation of fluid in the subcutaneous tissue due to impaired lymph drainage (Lambert, 2005).

Studies have revealed the presence of an endosymbiont, *Wolbachia* in arthropods and nematodes (Prakash and Puttaraju, 2007; Bouchery *et al.,* 2012). *Wolbachia* is a Gram-negative intracellular α-proteobacterium belonging to the Order *Rickettsiales* (Bouchery *et al.,* 2012). The species

Wolbachia pipientis was first identified in *Culex* mosquito (Hertig *et al.*,1924; Porennan *et al*.,2008). It is classified into sixteen sub groups (A-Q) (Guan *et al.*, 2016). Supergroup G have been removed because its status is currently not clear (Baldo and Werren, 2007). Of the sixteen sub groups, C and D are commonly found in filarial nematodes (Werren *et al.*, 2008), while A and B are found in mosquitoes (Ravikumar *et al*., 2011). According to Dyab *et al.*, (2016), *Wolbachia* are maternally inherited and infect a wide range of insects and nematodes and play an important role in the development and pathogenesis of the filariae *Onchocerca volvulus, Brugia malayi and Wuchereria bancrofti*. *Wolbachia* as a parasite, relates with its host in a number of ways, some of which are parasitism while others are mutualism. The parasitic relationship includes feminization of genetic males, pathenogenetic induction resulting in the development of unfertilized eggs, the killing of male progeny from infected females, and sperm-egg incompatibility (Werren *et al.*, 2008). *Wolbachia* appear to contribute to the inflammation process which is a major pathological symptom of filarial infections (Dyab *et al.*, 2016). The most common effect of *Wolbachia* infection in mosquitoes is cytoplasmic incompatibility. Mating of infected male with the uninfected female mosquitoes of the same species, does not result into fertilization (Sinkins, 2004). *Wolbachia* has the potential to be used as biocontrol in both pest and in biomedical applications (Zabalou *et al*., 2004; Engelstadter and Hurst, 2009;). Recombination between strains in *Wolbachia* affects various regions of the genome including surface proteins genes such as Wsp genes (Foster *et al*., 2011;Pinto *et al*., 2012; Uday and Puttaraju, 2012). Genetic and functional diversity among bacteria is greatly accelerated by recombination and thus enable bacterial adaptation and the Wsp protein is likely to mediate interaction with the host cell. (Werren *et al.*, 2008).

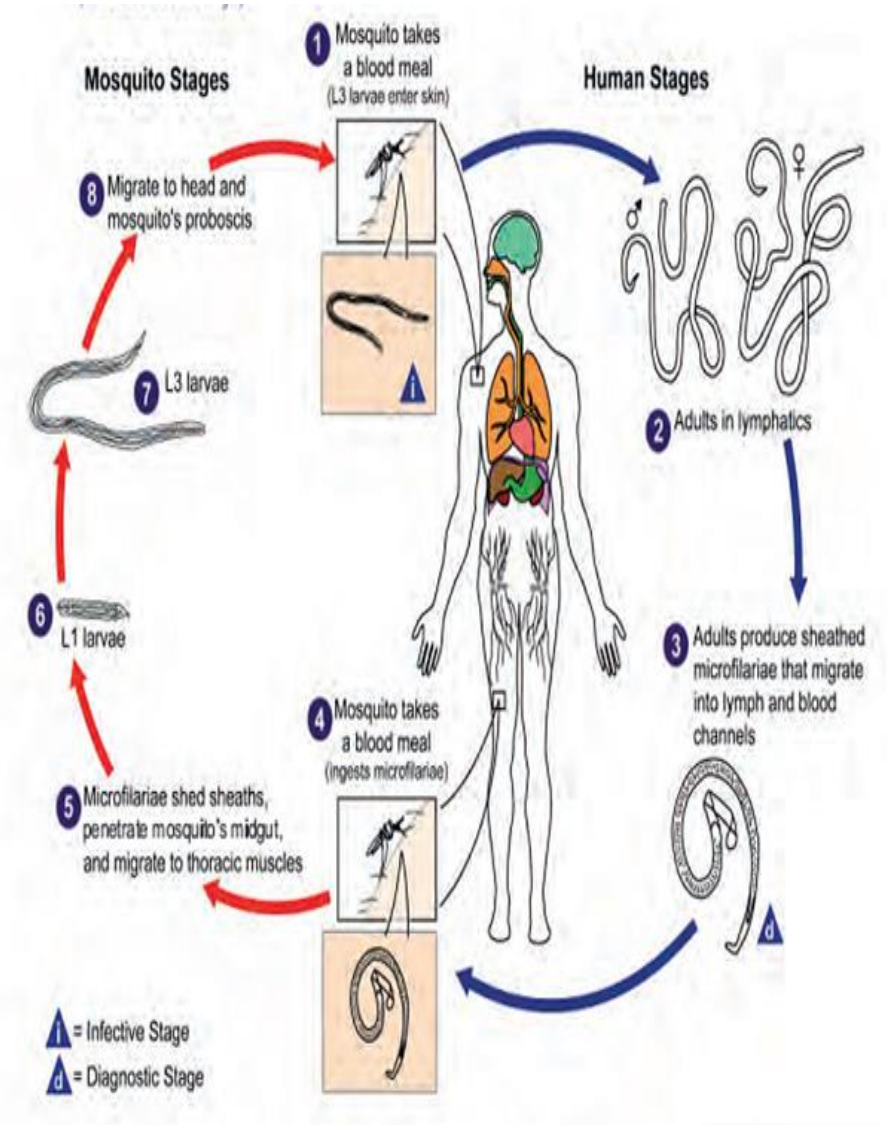


Figure 1.1. The life cycle of *W. bancrofti* (Singh *et al*., 2013)

1.10. Stages of the life cycle of filarial nematode (*W. bancrofti*)

Stage 1

In this stage, mosquito carrying an infectious microfilariae (L3) stage in its proboscis takes a blood meal from a person. In the process, the L3 larvae enters into the blood vessels of the person through the wound.

Stage 2

The L3 larvae migrate into the lymphatic and develop into mature adults. When mature , the female adults has a length of 80 to 100 mm and a diameter of 0.24 to 0.30 mm. The male adults measures 40 mm in length and 1 mm by diameter.

Stage 3

Mating occurs in this stage and the eggs hatch into microfilariae in the female. The sheathed microfilariae which are 244 to 296 mm by length and 7.5 to 10μm by diameter are released from the fecund adult female worms into the blood circulation of a person. One adult female can release up to 10,000 microfilariae per day. These microfilariae are mostly nocturnal periodicity. The microfilariae spend the day in the lymphatic vessels and spend the night in the peripheral circulation.

Stage 4

The microfilariae are more abundant in the circulatory system at night. During blood meal, the night biting mosquitoes ingest these microfilariae.

Stage 5

In the intermediate host (mosquito), the microfilariae lose their shealth and move down through the walls of the proventriculus and cardiac portion of the mosquito midgut and finally to the thoracic muscles.

Stage 6

In this stage the microfilariae develop into L1 larval stage

Stage 7

The microfilariae at this stage reach the infective stage (L3)

Stage 8

Here the infectious larvae travel through the hemocoel to the mosquitoes proboces. When the mosquito bites another person during a blood meal, the infectious larvae will enter the blood stream of the person and the cycle starts again.

1.2 STATEMENT OF RESEARCH PROBLEM

Lymphatic filariasis has greatly affected the daily activities of the infected people. Its impact on marriage and sexual life in endemic areas cannot be underestimated (Nwoke et al., 2010) Women who depend heavily on their physical appearance more than men for self-esteem (World Bank, 1993); are more of a disadvantage due to lymphedema and elephantiasis which seriously deform their appearance and permanently place them at a disadvantage in terms of opportunities for marriage (Nwoke et al., 2010). The Gambia was among the countries with the highest prevalence of lymphatic filariasis in Africa in the 1950s, with 50% of the adult population being reported positive for microfilariae (Hawking, 1957). In 2013, the World Health Organization listed The Gambia as among 73 countries considered endemic for lymphatic filariasis (WHO, 2013). Senegal which borders Gambia in the East, North and South except the western part has a prevalence of clinical manifestation of 14% of lymphatic filariasis (Faye *et al.*, 2009). According to Cano *et al.,* (2014), Global Atlas of helminthes infection, Gambia still has a high probability of transmission. Recent surveys conducted revealed a massive reduction in lymphatic filarial

endemicity (Rebollo *et al.*, 2015). The Gambia relies heavily on tourism and the presence of lymphatic filariasis has the potentials to reduce the number of tourists intending to visit the country. The warning travellers alert on the existence of lymphatic filariasis in the Gambia is still on the travellers notification 2016 website.

In the Gambia, transmission rate of lymphatic filariasis was said to be 0 % and was attributed to the extensive use of insecticidal nets for malaria control for decades since mass drug administration (MDA) was never implemented in the fight against lymphatic filariasis. (Rebollo *et al.*, 2015). Malaria which is also vectored by anopheles mosquito remain endemic in The Gambia thus the assumption of the use of insecticidal net of being responsible in preventing transmission of lymphatic filariasis as mentioned by Rebollo *et al*., (2015) may not be the case. Some strains of *Wolbachia* are known to protect their host against human parasites which led to the suggestion that *Wolbachia* –infected mosquitoes could be used to prevent transmission of human parasites from infected mosquito species (Jewelna *et al*., 2012). The incidence of *Wolbachia* in anthropophilic mosquitoes in The Gambia has not been previously reported.

1.3 JUSTIFICATION OF STUDY

Many species in the genera *Anopheles, Culex* and *Aedes* are well known for transmission of infective larvae that cause lymphatic filariasis. *Anopheles* and *Culex* are the most abundant species of mosquitoes present in the Gambia. They are both known for being the vector for lymphatic filariasis in the world. Lymphatic filariasis is mainly characterized by the occurrence of inflammatory pathogenesis, and *Wolbachia* bacterium which is an endosymbiont of filarial

nematode appear to contribute to this inflammation (Barton *et al*., 2010; Claudio *et al*., 2012 Bouchery *et al*., 2013).

Wolbachia mainly infect the reproductive tissues of arthropods and are vertically transmitted through the egg cytoplasm and alter their host reproduction in various ways (Puttaraju and Prakesh, 2009). The endosymbiont *Wolbachia* could be genetically transformed to modify the disease transmitting abilities of mosquitoes (Yeap *et al*., 2011; Fraser *et al*., 2017). It is crucial to know which *Wolbachia* strains are present in population before releasing engineered infected individuals because pre-existing natural infections can interact with and alter the dynamics of introduced strains (Duron *et al*., 2010; Atyame *et al*., 2011). Understanding the role of wolbachia in filarial transmission can provide a template for breaking the transmission chain of the disease.

There is also no data on their prevalence. Lymphatic filariasis is a disease without border, complete eradication in one country does not preclude its re-emergence and migration of people from one country to another can easily introduce or re-introduce the disease.

1.4 AIM

To screen mosquitoes in the Gambia for the presence of filarial nematode and *Wolbachia* using molecular techniques

1.5 SPECIFIC OBJECTIVES

i. To determine the distribution of various anthropophilic mosquito genera in The Gambia using G' ruumba method and microscopy.

ii. To identify *Wolbachia* in infected mosquitoes in The Gambia using polymerase chain reaction technique

iii. To characterize filarial nematodes found in infected mosquitoes in The Gambia using polymerase chain reaction technique

1.6 HYPOTHESIS

Ho: Anthropophilic mosquitoes in The Gambia are not infected with *Wolbachia* and filarial

CHAPERT TWO

2.0 LITERATURE REVIEW

2.1 FILARIASIS

Lymphatic filariasis is the World second leading permanent and long term disability (Maldjian *et al*., 2014). It is a neglected tropical disease (NTD) affecting 120 million people in 83 countries. In The Gambia, the fight against malaria may have interrupted active transmission of lymphatic filariasis (Rebollo *et al.,* 2015). According to Gyapong *et al*., (2013), lymphatic filariasis caused by *Wuchereria bancroftic* in The Gambia was among the highest in Africa with a prevalence of 50% of adult population carrying the microfilariae.

2.2 SIGNS AND SYMPTOMS OF LYMPHATIC FILARIASIS

Infection mostly occur during childhood but it takes a longer period for clinical symptoms and manifestation to be observed. The symptoms could be divided into two:

2.2.1 Physical symptoms

- Pain from the affected part
- Swollen limbs
- Bad smelling lesions in the skin
- Enlargement of the arms, legs, breast, or genitals
- Difficulty in walking and mobility
- Damage to the lymphatic system and kidney
- Chronic lymphatic filariasis will show : hydrocele (swollen scrotum), acute epididymitis and funicalitis

- Chyluria with loss of fat –soluble vitamins and protein resulting in malnutrition and vitamin deficiencies
- Tropical eosinophilia syndrome which is associated with cough, shortness of breath and weezing. (WHO, 2017)

2.2.2 Psychological symptoms

- Personal shame
- Lost / diminished economic productivity
- Becoming a burden to the family and the community
- Social loss in the form of broken friendships and marriages (WHO , 2017)

2.3 SOCIAL IMPACT OF LYMPHATIC FILARIASIS

Lymphatic filariasis with clear clinical manifestation such as lymphedema of the limbs, breast and external genitilia pose a high degree of stigmatization which detached the individual from all forms of social gathering (Upadhyayula *et al*., 2012; WHO, 2017). The infected individual due to shame may tend to avoid seeking treatment which could lead the disease condition to chronic levels (Upadhyayula *et al*., 2012). The burden is more severe in women who depend greatly on their physical appearance more than men for self-esteem (World Bank, 1993; WHO, 2017). The disease manifestation could lead to broken marriages for those who are already in a relation while those unmarried young women may have diminished prospect of getting husband (CDC, 2013). In Thailand and in West Africa, there is a general beliefe that children born to a woman affected by lymphatic filariasis will be similarly affected (Omudu *et al*., 2010). The infected women could be perceived by men as financial burden if married (Krishna *et al*.,2005; Omudu *et*

al., 2010). The disease condition may prevent the infected individuals from taking part in the labor force (WHO, 2017). According to Otabil *et a*l., (2015), lymphatic filarial infected individuals with hydrocele find it difficult to establish their sexual identity as well as lack the potentials to carry the economic burdens when married. Other social problems associated to lymphatic filariasis are marriage without sexual activities , report of painful intercourse in women whose partners had penile lymphedema (Streit and Lafontant, 2008; Tejaswini *et al*., 2017). Acute cases of idenolymphadenitis in children could result into school absenteeism and poor educational attainment (Wynd *et al*., 2007)

2.4 EPIDEMIOLOGY OF LYMPHATIC FILARIASIS

The global distribution of lymphatic filariasis is to a larger extend determined by the carrier vector. According to Cano *et al*., (2014), 72 countries are currently endemic worldwide (Fig.2.1). The distribution of lymphatic filariasis is not even but restricted to certain localities in the World (Cano *et al*., 2014). In African continent, prevalence is more concentrated in West Africa than in Central, Eastern and Southern Africa (Cano *et al*., 2014). A third of the World lympahtic filarial infected persons live in Africa (WHO, 1993; Smith *et al*., 2017). Asian pacific Islands and Papaua New Guinea are also burdened by lymphatic filariasis with areas such as South and South-East and the Pacific being the epitome of the disease transmission (Cano *et al.*, 2014). Another one third of the World lymphatic filarial patients live in India (Smith et al., 2017). Although the environmental condition in the Central and South America may be suitable for transmission, active transmission is isolated and only reported in certain areas (Cano *et al*., 2014).

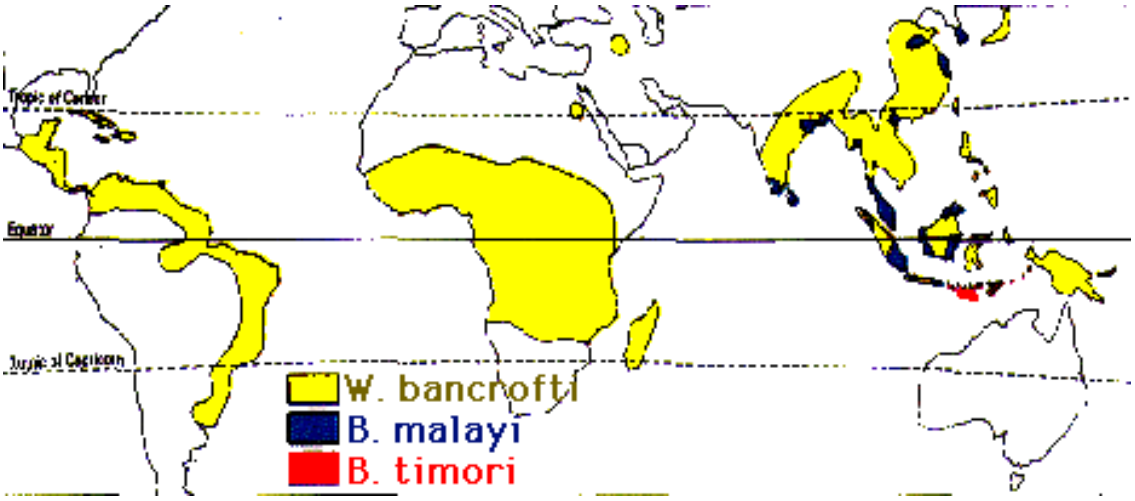


Fig. 2.1: Global distribution of lymphatic filariasis high lighting the types of lymphatic filarial parasites

Source: http:// www.nemaplex.ucdavis.edu (2012)

2.5 CLASSIFICATION AND MORPHOLOGY OF LYMPHATIC FILARIAL WORM

Lymphatic filariae are essentially of three species

2.5.1 *Wuchereria bancrofti* (*W. bancrofti*)

Phylum : Nematoda

Class : Secernentea

Order : Spirurida

Family : Filaridae

Genus : *Wuchereria*

Species : *W.bancrofti*.

The microfilariae of *W,bancrofti* are between 250 to 300µm in length while the mature worm could be as long as 30 to 100mm with a diameter of about 100 to 300µm (Ciocan *et al*., 2010). The males are generally smaller than their female counterparts. The adult *W.bancrofti* has a smooth cuticle with one end being blunty while the other end is pointed. The absence of terminal nuclei at the tail region clearly distinct it from the *Brugia* genus. Like *Brugia* genus, *W. bancrofti* lacks a digestive system, nutrients are absorbed directly from its host.(David *et al*., 2006) Although *W. bancrofti* is vectored mainly by *Anophelia* mosquito, it has a more vector diversity than *B. malayi* (Ciocan *et al*., 2010).



Plate 2.1. Structure of *Wuchereria. bancrofti*

Source : http**://** **www.mcdinternational.org** **(2017)**

2.5.2 *Brugia malayi (B. malayi*)

Phylum : Nematoda

Class : Secernentea

Order : Spirurida

Family : Onchoceridae

Genus : *Brugia*

Specie : *B. malayi*

Unlike *W. bancrofti*, *B. malayi* has a round anterior and a pointed posterior end (WHO, 1997). The microfilariae of *malayi* are 200 to 275µm in length with sheathed body like *W. bancrofti*. They have two separate nuclei which extend to the tail region (David *et al*., 2006). The presence of *B. malayi* in monkeys, cats and dogs, indicate that host specificity is greater in *B. malayi* than in *W. bancrofti* and *B. timori* (Ciocan *et al*., 2010). Microfilariae of *B .malayi* can be found in the peripheral blood during the day. The vectors are mainly day biting mosquito, mostly *Aedes* (David *et al*., 2006). The pathology of *B. malayi* differs in some ways with *W. bancrofti* in that *B. malayi* does not readily exhibit swelling of the genital organ or chyluria as well as expression of elephantiasis is limited at the lower limbs (David *et al*., 2006).

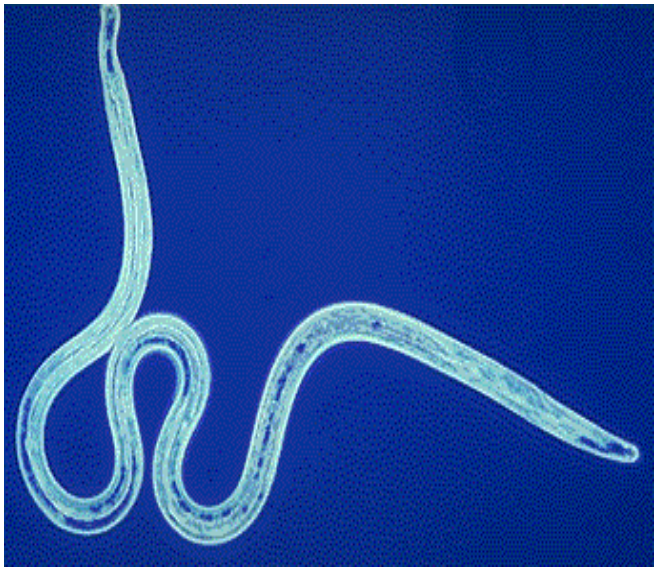


Plate 2.3. Structure **of** *Brugia malayi (B. malayi)*

Source: http:// nematode.net (2017)

2.5.3 Brugia timori (B. timori)

Phylum : Nematoda

Class : Secernentea

Order : Spirurida

Family : Onchoceridae

Genus : Brugia

Specie : *B. timori*

Brugia.timori resembles *B. malayi* while in the case of vector association, it resembles *W. bancrofti* by having anopheles as its vector (David *et al*., 2006). Its pathogenesis is similar to *B. malayi*.



Plate 2.3: Structure of *Brugia timorii (B. timori)*

Source: http://xyala2.bio.ed.ac.uk (2017)

2.6 LYMPHATIC FILARIASIS VECTORS

The mosquito is the major vector involved in filariasis transmission. According to WHO (2011), Lymphatic filariasis is the first mosquito borne disease to be discovered. The mosquito family is divided into two subfamily: the Culicidea, which consist of *Anopheles gambiae, Anopheles funestus, arabiensis* and the subfamily Culicinea which includes *quinquefasciatus, pipiens, tarsalis* and *salinarius* (Ozioma *et al*., 2015).

According to Ozioma *et al.,* (2015), mosquito borne disease pose risk to 50% of the World population. Diseases such as malaria, dengue fever, lymphatic filariasis, yellow fever, St Louis encephalitis etc, are vectored by mosquitoes of different genera. The adult mosquito is about 2mm to 12.5 mm in length. Their feeding habits differ from genera to genera. Some genera bites only during the day time while others bite in dawn and dusk , yet others are more active at night. Generally, adult mosquitoes of both sexes feed on nectar and plant juice for their energy requirements .The female mosquitoes for the purpose of reproduction, need a blood meal from mammals and birds to help develop their eggs and in the process, the disease causing agents are introduced into the host system for which the disease is intended for (Minakawa *et al.*, 2005). The male mosquitoes differs from females by the presence of bushy bristles on the antennae. The male mosquitoes live a shorter life span of about two weeks while their female counterparts live for four weeks or even months. Generally, when an adult male mosquito fertilizes the female, the male dies and the female too dies when it finishes laying its eggs. The eggs take a period of about 2-3 days to hatch into larvae depending on temperature (Harbach, 2013). The larval stage mimics active life , it feeds and swims from place to place while the pupal stage which last for

two to seven days dose not feed Lymphatic filariasis is vectored by four genera of mosquitoes : *Anopheles, Culex Aedes* and *Mansonia*.

2.6.1 The Genus: *Anopheles*

According to Harbach, (2013), 430 species of the *Anopheles* genera exist and distributed in both tropical and temperate regions of the World. Like other genus, Anopheles has a well-defined metamorphosis (egg, larva,pupal and adult). The larvae of *Anopheles* mosquitoes lack siphons character. The eggs are boat-shape. The palps are as long as the proboscis (Foster and Walker, 2009). *Anopheles* mosquito can be differentiated from other genera by their resting position. Their abdomen are always raised in the air while resting (Foster and Walker, 2009). The activities of *Anopheles* mosquitoes are nocturnal (Harbach, 2013). Although habitat of mosquitoes to a large extend varies from specie to specie, some species have adaptation skills which enable them to cope in different environments. Traditionally, *Anopheles* mosquitoes are restricted to clean or clear water environment for breeding. However certain species such as *Anopheles gambiae* are said to be adapted to polluted water environment for breeding (Geissbuhler, 2008; Laporta *et al*., 2011). Increasing surface water pollution which is a characteristic feature of urbanization, has unintentionally eliminated *Anopheles funestus* mosquito specie from such environments (Geissbuhler, 2008; Laporta *et al*., 2011). Generally, *Anopheles* genera of mosquito larvae could be found in fresh water, salty water, marshes, mangrove swamps, rice fresh, grassy ditches, the edges of streams and rivers and small temporary rain pools (Geissbuhler, 2008).

2.6.1.1 Pathogenicity

Anopheles genus is implicated in the transmission of numerous diseases worldwide. The key among the diseases being transmitted is malaria (Paulo *et al*., 2015). Other diseases transmitted by the genus *Anopheles* are: dog heartworm (Ledesman and Harington, 2011), filariasis (Dziedzom *et al*., 2012) and dirofilariasis (Cancrini *et al*., 2006),



Plate 2.4: *Anopheles* mosquito

Source: https:// commens.wikimedia.org (2011)

2.6.2 The Genus *Culex*

Culex mosquito genera belongs to the subfamily Culicinae. More than 200 species of *Culex* mosquitoes have been reported (Harbach, 2012). The adult culex is about 3 to 4mm in length and the palps are unequal to the proboscis in length. *Culex* mosquitoes are grey-black in colour. Their eggs are laid glued together into a raft. The larvae hang beneath water surface at an angle. The presence of siphons enables culex genera access to oxygen on the water surface. Just like anopheles mosquitoes, *Culex* mosquitoes prefer permanent water bodies for breeding (Lun *et al*., 2012). They lay their eggs on the surface of stagnant water bodies at the edges of lakes and ponds in the vegetation of swamps and marshes (Lun *et al*., 2012). Although clean water bodies are preference for some genera of *Culex* mosquitoes, some other species such as quinquefasciatus are turbid/ polluted water favorites (Lun *et al*., 2012).

2.6.2.1 Pathogenicity

The genus *Culex* is implicated in the transmission of a number of diseases some of which are deadly. Diseases such as: West nile virus (WHO, 2011; Tonya *et al*., 2012), dog heartworm (Ledesman and Harington, 2011), Filariasis (Dziedzom *et al*., 2012), dirofilariasis (Cancrini *et al.*, 2006), Japanese encephalitis (WHO, 2015), Saint Louis encephalitis (Warrell and Mclellen, 2013), Western equine encephalitis (Mohan *et al*.,2016; Phelps., *et al*., 2017), Venezuellan equine encephalitis (Derlet, 2016; Shaw *et al*., 2016) and barmat forest fever (Naish *et al*., 2006; Naish *et al*. 2011) are also transmitted by Culex.



Plate 2.5: *Culex* mosquito

Source: https://pixnio.com/fauna-animals (1996)

2.6.3 The genus: *Aedes*

This genera of mosquito consist of 950 species of which some are carriers of disease pathogens while others cause nuisance by biting humans and other animals (Nazri *et al*., 2013). Generally, they have a small body size compared to the other genera of mosquitoes. They are easily recognizable by their black body and zebra striped patterns of black and white on their legs (Nazri *et al*., 2013). On resting position, their bodies are kept parallel to the surface with their proboscis angled downwards (Nazri *et a*l., 2013). They have pointed abdomen compared to *Culex. Aedes* genera are inhabitants of temperate and tropical regions of the World. Like the Culex, Aedes larvae hang beneath the water surface with the surport of the siphons for oxygen collection. *Aedes* genera of mosquito are specialized day biting mosquitoes. Different species in that genera can transmit the same disease pathogens. For example *A. aegypti* is the principal vector for dengue fever (Yukiko, 2011), the disease can also be transmitted by *A.albopictus* (Carington and Simmons, 2014). Aedes mosquitoes are basically indoor lovers. They spend their lifetime in or around houses, where they rest and feed on human blood during the day (Bhatt *et al*., 2013; Samantha and Christopher, 2014). They lay their eggs in containers holding water bodies, stagnant rain water and discarded tires which hold rain water (Dejener *et al*., 2015).

2.6.3.1 Pathogenicity

Aedes genera has been a vehicle in the transmission of many diseases one of which is the causative agents of the deadly zika virus (Rengina, 2016). Other diseases transmitted by *Aedes* genera are: Denque fever (Yukiko, 2011; WHO, 2017), Chukungunya (Lack *et al*., 2014; WHO, 2017), Dog Heartworm (Ledesman and Harington, 2011), Yellow fever (WHO, 2016), Filariasis (Dziedzon *et al*., 2012), Tularemia (Jeannine *et al*., 2009), Western equine encephalitis (Mohan *et al*.,2016; Phelps *et al.*, 2017), Eastern equine encephalitis (Armstrong and Andreadis, 2010), Venezuella encephalitis (Derlet; 2016; Shaw *et al*., 2016) and Barmat Forest fever (Naish *et al*., 2011). The genera *Aedes* seems to be the most specialized mosquito in disease transmission. This is evident by the number of disease *Aedes* has been implicated in the transmission.



Plate 2.6: Aedes mosquito

Source: http://public health.yale.edu (2011)

2.6.4 The genus *Mansonia*

Mansonia mosquito belongs to the subfamily 'Culicinae' with two subgenera: '*Mansonia*' subgenera which consist of 15 species and the '*Mansoniini*' subgenera consisting of 10 species. The *Mansonia* genus is implicated in the transmission of various arboviruses and lymphatic filarial nematodes (Ughasi *et a.l*., 2012) While the subgenus *mansoniini* has a profile of transmitting *B.malayi* in India and Southeasten Asia, the subgenus *Mansonia* commit itself in the transmission of *W. bancrofti* in West Africa, Sourthen Asia, Australia and Japan. The *Mansonia* genus is characterized by black body colour with a mixture of dark and pale scales giving it a sparkling appearance on its wings and legs (Stone *et al*., 1959)

2.6.4.1 Pathogenicity

Mansonia genera seem to be the least mosquito species implicated in disease transmission. Of all the mosquito borne diseases, *Mansonia* is reported to have taken part in the transmission of only filariasis (Dziedzom *et al*., 2012) and Venezuella encephalitis (Derlet, 2016; Shaw *et al*., 2016).



Plate 2.7: *Mansonia* genera

Source: http// knowledgebase_ lookseek.com (2005)

2.6.5 LIFE CYCLE OF MOSQUITO

The life cycle for all the genera of mosquitoes are of four distinct stages; Egg, Larvae, Pupae and Adult.

2.6.5.1 Egg stage

Mating occurs between adult male and female mosquito once in their lifetimes**.** Once mating occurs**,** the female will lay 100 to 300 eggs every blood meal in every three days. The eggs are laid in clumps in the form of " rafts " or floating on the surface of stationary water bodies, on the ground , in pots or any other area that regularly floods depending on genera of mosquitoes. (http://www.epa.gov/mosquito control)

2.6.5.2 Larval stage

This is the next stage in the life cycle after egg stage. The egg hatch into larvae called "wrigglers which hang on the surface of water bodies using their tubes to breath from the surface The larvae feeds on organic matter remains from aquatic life. This stage last for a week.

2.6.5.3 Pupal stage

The larvae after about a week, metamorphasise into pupae. The process of turning into pupae involves shedding their skin for about four times to get into a pupal stage. At the stage of pupal, called "tumblers" the young growing mosquito does not feed. The only activites the tumblers can do is to swim from the surface of the water to the deepest part of the water especially when threatened by predators. The tumblers have a comma shape by structure which are partially covered by a structure called cocoon with the head at one end while the other end is characterized by structures called flippers of which the pupae used in swimming. This stage last for about four days (http://www.epa.gov/mosquito control).

2.6.5.4 Adult stage

After about four days of a successful pupal stage, the cycle enters the adult stage. The new adult is ready to emerge from the waters for a new adaptation to life on land. It cripples and climb out of the water on to the land or other surfaces, rest and wait for its body to dry and fully start it new life as adult mosquito. The male mosquito takes about a day or two for its reproductive system to mature. The wingbeats of the females attracts the male mosquitoes for mating. (http://www.epa.gov/mosquito control).

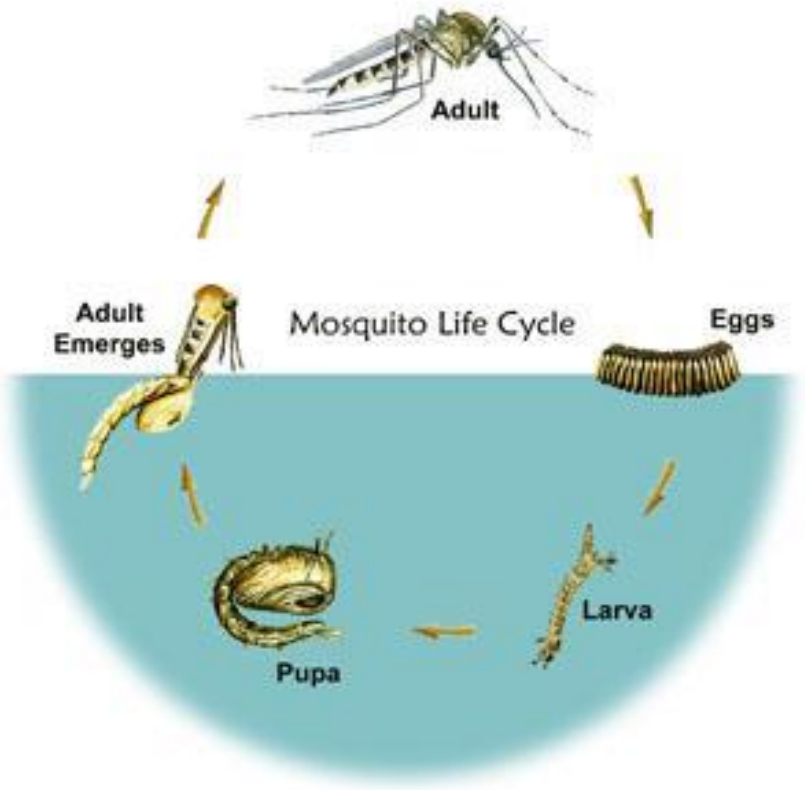


Plate 2.8 The life cycle of a mosquito

Source: http://www.epa.gov/mosquito control (2017)

2.7 DIAGNOSIS OF LYMPHATIC FILARIASIS

The causative nematode of lymphatic filariasis, the microfilariae can be detected from its vector (mosquito) and from the human host using several detection methods such as microscopy, serological technique (Antibody detection), molecular technique

2.7.1 Microscopical detection method

Microfilariae could be detected using microscopy by examining the circulatory microfilariae in the peripheral blood smear samples of patients and from dissected insect vectors (Samantha *et al.,* 2012). Processes such as centrifugation and filtration can increase the sensitivity of the method (Dyab *et al.,* 2016). Asymptomatic cases of lymphatic filariasis are better detected when peripheral blood smear are screened in low power lense microscopes (Sneha and Chawarkar, 2016).

In addition detection of larvae could be done by dissecting the insect and visualizing with a microscope (Samantha *et al*., 2012). Differentiation of *Bancroftian* and *Brugian* filarial nematode by microscopy is based on a number of morphological features such as size, density of the nuclei and appearance of nuclei on the tail end of the nematode. Bancroftian nematodes are generally smaller than their Brugian counterparts with the absence of nuclei at the tail (Sneha and Chwarkar, 2016). Detection of larvae from the mosquito could suggest the specie of filarial nematode since each species of filarial nematode is vectored by a different genera of mosquito (Samantha *et al*., 2012).

2.7.2 Serological technique (Antibody detection)

The presence of a causative organism in a host triggers the host immune system to produce antibodies in an effort to combat the invading agent. Lymphatic filariasis could be detected through ELISA antigen – antibody interaction. Antigens have been designed to recognize and bind to the already circulating antibodies which are produced as a result of the presence of filarial causative organism (Samantha *et al*., 2012). For instance wb-sxp-1 and Bm-sxp-1 recombinant filarial antigen specific to particular antibodies have been constructed to be used in a sandwich ELISA for the detection of circulating filarial antigens in sera from patients of *W.bancrofti* and *B. malayi* (Noordin *et al*., 2010). The challenge of this method is that the tendency for a cross-reaction between antigens of the three lymphatic filarial species as well as the other filarial specie is inevitable, thus weakening the sensitivity in differentiating the type of filarial nematode present (Weil *et al*., 2011; Hall and Dogcio, 2016). The method could give false positive in cases where the microfilariae are dead while the results indicate current infection (Noordin *et al*., 2010).

2.7.3 Molecular detection technique

Molecular technique of detection of filarial DNA is based on the presence of short tandem repeat sequence existing in the DNA of the filarial nematode. These repeat sequences are mostly specie specific (Samantha *et al*., 2012). The method can be used to detect the presence of the filarial nematode in the host human blood and in mosquito vectors (Noordin *et al*., 2010).

2.7.3.1 Polymerase chain reaction targeting Ssp1

The Ssp1 is a tandemly repeat in the conserved region with 188bp used as target in the PCR detection for *W. bancrofti* (Siridewa *et al*., 1996). The forward primer for the SspI gene of *W. bancrofti* is SspI F 5'-CGT GAT GGC ATC AAA GTA GGG-3', and the reverse primer for the SspI gene is SspIR 5'-CCC TCA CTT ACC ATA AGA CAAC-3'. The sensitivity of detection of microfilariae from mosquito using PCR can be more if the mosquitoes are dried, crushed and homogenized before extracting DNA (Vasuki *et al*., 2001).

2.7.3.2 Polymerase chain reaction targeting Hha1

Hha1 is a repeat sequence in *Brugia* genome and a PCR based diagnostic target for *Brugia malayi* yields an amplicons size of of 320bp (Vasuki *et al*.,2001; Noordin *et al*., 2010;). In a study conducted by Noordin *et al*., (2010), 66 out of the 124 blood samples which tested positive for microfilariae microscopically, were also found to be positive with PCR using Hha1 species specific primers: HhaI F 5'-GCG CAT AAA TTT ATC AGC-3' and the reverse primer was HhaI R 5'- GCG CAA AAC TTA ATT ACA AAA GC-3' Furthermore, the PCR based diagnoses were able to detect four positive samples out of the 30 samples which were pronounced negative from microscopic analysis (Noordin *et al*., 2010). This is a clear indication of the specificity of the Hha1 PCR diagnostic for Brugia against the traditional microscopic diagnosis.

2.7.3.3 Polymerase chain reaction—restriction fragment length polymorphism analysis

Internal transcribed spacer (ITS1) gene is situated between the small-subunit ribosomal RNA (rRNA) and large subunit ribosomal RNA (rRNA) gene in the chromosome. This gene occurs in

tandem repeats. (Gilles *et a*l., 1998). This gene is easy to detect even from small quantities of DNA due to its high copy number of the rRNA clusters (Balwin *et al*., 1995). Its high degree of variation even between closely related species gives it an adavantgae to be used as a target in diagnosis.(Jingyuan *et al*., 2012). The PCR-RFLP of the ITS1 gene has a greater potentials to differentiate filarial nematodes to species level especially in situations of co-endemicity (Surang, 2009). In a study conducted by Surang, (2009) to detect and differentiate filarial nematodes into species levels, primers FL1-F(5'-TTCCGTAGGTGAACCTGC-3') and FL2-R (5'-ATATGCTTAAATTCAGCGGG-3') in 18S and 28S ribosomal DNA respectively were used to amplify ITS1/5.8S/ITS2 fragment from total nematode DNA, the products were subjected to a digest using the Ase restriction enzyme. The fragments are then analyzed using gel electrophoresis and viewed under a UV light.

2.8 MANAGEMENT AND CONTROL OF LYMPHATIC FILARIASIS

2.8.1 Treatment

Lymphatic filariasis cause by *W. bancrofti*, *B. malayi* and *B. timori* are important tropical disease causing considerable morbidity. The management and treatment of this disease is futile for the fact that there are no vaccine for it at present coupled with the increase in insecticide resistance on the vectors. The current drugs in circulation are only capable of eliminating the microfilariae and not the macrofilariae. In other words, the existing drugs can only reduce active transmission and pathology and not to cure the patients from the disease conditions (Bockarie and Deb, 2010). The drugs currently in circulation on lymphatic filariasis are albendazole, Diethylcarbamazine (DEC) and Ivermectin (Fischer *et al*., 2017).

According to WHO, (2017), a single dose of diethylcarbamazine is as effective as the formerly recommended 12-day regimen. A single dose of Ivermectin (between 100 to 440µg/kg) has the potential to clear microfilariae of *W. bancrofti* and *B. malayi* from the blood (Fischer *et al*., 2017) but not effective on the adult worms. A single dose of Ivermectin in lowering the number of circulating microfilariae is as effective as the traditional 14 –days regimen of Diethylcarbamazine (DEC) (Fischer *et al*., 2017). Ivermectine has lower side effects compared to Diethylcarbamazine. However the sustainability of both Ivermectin and Diethylcarbamazine in reducing microfilaremia remain an issue (Fischer *et al.,* 2017). A combination of albendazole and ivermectin or diethylcarbamazin is 99% effective in clearing the microfilariae from the blood (WHO, 2017).

Wolbachia supergroup C and D infects filarial nematodes (Fenn *et al.,* 2006; Panaram and Marshall, 2007). The filarial nematode depends on wolbachia for its survival and reproduction (Barton *et al*., 2010). Therefore, an attempt to eliminate wolbachia in nematode will result in killing the nematode as well. Recent studies has recognized the obligate wolbachia endosymbiont as a drug target for anti-filarial nematode chemotherapy (Barton *et al.*,2010). The antibiotics: Doxyclin and Rifampicin are made to target wolbachia endosymbiont of filarial worm (Hoerauf *et al*., 2008; Mand *et al*., 2009). However, the long term duration for the treatment and its contra- indication in children under eight years, pregnant and breast feeding women reduces its applicability (Hoerauf *et a*l., 2008; Mand *et al*., 2009).

A new approach in combating the disease is using RNA interference in determining gene function and degrading the mRNA in an attempt to silence the gene (WHO, 2017).

Patients with clear clinical manifestations can only be managed to lessen the disease conditions since the available drugs cannot eliminate the macrofilariae (adult worms) from the lymphatic system. Proper management of the conditions includes daily washing with soap, the affected limbs to prevent secondary infections (WHO, 2017). Patients with hydrocele could be managed by surgical procedures (WHO, 2017). Simple exercise and elevation of the affected limb at night as well as mobilizing the affected limb with a bandage will greatly help in managing the conditions (Brian, 2017; Katarak *et al*., 2017). Diet of patients with lymphatic filariasis should be made deficient with fatty foods since lymphatic filarial patients often suffer from chyluria (Brian, 2017; Katarak *et al*., 2017)

2.8.2: Prevention and control of Lymphatic filariasis

This is often addressed in one of the following areas:

- Avoidance in coming contact with the vectors through personal or community measures, such as using mosquito repellants, sleeping under mosquito treated nets as well as clearing the environment to avoid mosquitoes from breeding (WHO, 2007)
- Habitual screening of the blood for all individals in endemic areas with microscope will help for an early detection and start medication to avoid more damage (WHO, 2017).

2.9 *WOLBACHIA* ENDOSYMBIONT

Domain : Bacteria

Phylum : Proteobacteria

Class :Alphaproteobacteria

Subclass : Rickettsidae

Order : Rickettsiales

Family : Anaplasmataceae

Genus : *Wolbachia*

Wolbachia are obligate alphaproteobacteria endosymbiont that infect arthropods and nematodes. (Hertig *et al*., 1924). *Wolbachia* was first described in 1924 by Hertig as *Wolbachia* pipientis. According to Guan *et al*.,(2016), sixteen supergroup of *Wolbachia* have been name (A to Q). Supergroup G have been removed because its status is currently unclear (Baldo and Werren, 2007).



Plate 2.9 *Wolbachia* endosymbiont

Source: http://wishart.biology.ualberta.ca (2004).

Table 2.1: Supergroup of *Wolbachia* , their hosts and relationship with hosts.

SUPERGROUP	HOST	RELATIONSHIP
A	Mosquitoes/Flies/Spiders	Parasitism
B	Mosquitoes/Flies/Spiders	Parasitism
C	Filarial nematodes	Mutualism
D	Filarial nematodes	Mutualism
E	Springtails	Not known
F	Arthropods and Filarial nematode	Not known
H	Termites	Not known

Adapted from (Werren *et al*., 2008)

Its adaptability in its host environment is empowered by the use of spindle apparatus during cell division (Shi *et al*., 2016) and the use of dynein and kinesin motors as means for movement within the host cells (Serbus and Sullivan, 2008; Shi et al., 2016). The locality of *Wolbachia* in its host is centered in the oocytes where some can be found in the germ pole of the eggs while others are found distributed in the egg cytoplasm and the resulting soma of the embryos (Veneti *et al.,* 2004).

Wolbachia induces cytoplasmic incompatibility in arthropods and distort the sex ratio (Bordenstein *et al*., 2001; Charlat *et al*., 2006; Telschow *et al*., 2007). *Wolbachia* infected females when mate with infected males produces offspring as well as *Wolbachia* uninfected females mate with uninfected males produces offspring (Bordenstein *et al*., 2001; Telschow *et al*., 2007). When uninfected females mosquitoes mate with infected males mosquitoes, no

offspring is produced (Bordenstein *et al*., 2001; Chalat *et al*., 2006). Introducing *Wolbachia* into a natural population will decrease pathogen transmission and reduce disease burden (Hancock *et al*., 2011)

2.10 *WOLBACHIA* AND ITS ARTHROPOD HOST

Unlike nematodes, wolbachia relates with their mosquito hosts in a more parasitic manner where they influence to a large extent the reproduction and development of the host mosquito (Barton *et al.,* 2014). According to Barton *et al* ., (2014), *Wolbachia* has the potential to be employed as a biocontrol due to its ability to interrupt the transmission of certain diseases such as dengue fever and also capable of preventing infectivity of malaria parasite and the filarial nematode as well as reducing the mosquito population through its cytoplasm incompatibility. Therefore, infecting mosquito population with *Wolbachia* could help in controlling many mosquito borne diseases Recent studies has also demonstrated that certain strains of *Wolbachia* could confer resistance to the plasmodium parasite in *Anopheles stephensi*, while wolbachia strain B interrupt the viability of filarial nematode in *Anopheles aegypti* (Barton *et al.,* 2014). Genes which codes for antiparasite effect can be inserterd into the vector or *Wolbachia* genome along with linked "cl genes" (cytoplasmic incompatibility) to prevent transmission of parasite (Barton *et al* ., 2014).

2.11 *WOLBACHIA* AND ITS NEMATODE HOST

Filarial nematode infects about 150 million people in the tropics. *Wolbachia* intracellular alphaproteobacteria is an obligate endosymbiont that infect nematodes and arthropods. The supergroup of wolbachia that infect nematodes are C and D. *Wolbachia* is reported to be

responsible for the pathogenicity and immune response in filarial infection (Genchi *et al*., 2012). *Wolbachia* provides its host with some chemicals necessary for its reproduction and survival (Grote *et al.*, 2017). Elimination of *Wolbachia* endosymbiont from its filarial nematode will cause death or sterility to the host nematode (Kent *et al*., 2011). The drugs used in the fight against lymphatic filariasis can only eliminate the microfilariae and thus the introduction of doxycline antibiotic to target *Wolbachia* instead of the filarial nematode (Genchi *et al*., 2012).

2.12 TRANSMISSION OF *WOLBACHIA*

Wolbachia is stationed within the cytoplasm of the cells of the infected mosquito (Bechmann *et al*., 2017). The spermatozoa of infected male mosquito undergoes modification such that the embryos dies during early embryonic development (Bechmann *et al*., 2017; Lepage *et al*., 2017). *Wolbachia* is maternally transmitted (vertical) from infected females to the offspring (Bechmann *et al*., 2017). Transmission of wolbachia can only occur if infected female mosquito mates with uninfected male or infected male mosquito. When uninfected female mosquito or female infected with a different strain of *Wolbachia* mates with infected male mosquito, no offspring is produced (Riparbelli *et al*., 2012).

2.13 DETECTION OF *WOLBACHIA* IN MOSQUITOES

2.13.1 Near infrared spectroscopy

It is a technique that uses the near infrared region of the electromagnetic spectrum (from about 700 to 2500nm) to measure specific frequencies of light absorbed by C---H, O---H, S---H and N----H functional groups in a sample (Sikulu *et al*.,2016). Differences in compositions and concentration of biological molecules results in difference in absorption differences in the near

infrared regions (Sikulu *et al*., 2016). The technique requires little or no sample preparation; it is non -destructive to the sample and takes only 15seconds to run a sample ((Sikulu *et al*., 2016). The use of near infrared in species identification is 35 times cheaper and over 16 times faster than PCR and the conventional microscopy method (Sikulu *et al.,* 2010). In a research conducted by Sikulu *et al*., (2016), mosquitoes infected with *Wolbachia* were differentiated with the wild uninfected with an accuracy of 96%. The results were confirmed using PCR to detect wsp gene (Sikulu *et al*., 2016).

2.13.2 Polymerase Chain reachion Technique

Various primers have been designed to target various conserved regions in the genome of *Wolbachia* endosymbiont. Among them 16srDNA is used to detect all supergroup of *Wolbachia* in mosquito genera while Wsp is used to classify *Wolbachia* into supergroup A or B (Table 2.2).(Badini *et al*., 2014; Dyab *et al*., 2016; Shaw *et al* 2016; Nugapola *et al*., 2017).

Wsp genes encording cell surface protein (CSPs), are believed to act in many important cell functions such as receptors, transporters, channels and enzymes (Cunha *et al*., 2009). They are also use as target in molecular diagnostic and therapeutic tools due to their subcellular localization (Cunha *et al*., 2009). *Wolbachia* surface protein gene; wsp encodes a major surface membrane protein showing sequence similar to major outer membrane protein of closely related alphaproteobacteria (Baldo et al., 2005). Wsp gene in *Wolbachia* is the most variable gene among the existing gene sequences of *Wolbachia* showing relatively high genetic divergence among strains (Pinto *et al*., 2012).

Wobachia surface protein (WSP) is an eight beta-barrel transmembrane structure which participate in host immune response, cell proliferation , pathogenicity and controlled cell death program (Uday and Puttaraju, 2012).

Table 2.2 Sets of primers for identification of *Wolbachia* supergroup A and B

Wolbachia/ Supergroup	Primers	PCR product (bp)
Wolbachia Genera	16srDNA (W-SPECF 5'CATACCTATTCGAAGGGTAC 3') 16srDNA (W-SPECR 5'AGCTTCGAGTGAAACCAATTC 3')	438
Group A	wsp 136F (5' TGAAATTTTAGCTCTTTTC 3') wsp 691R (5' AAAAATTAAACGCTACTCCA 3')	556
	wsp 308F (5' CCAGCAGATACTATTGCG 3') wsp 691R (5' AAAAATTAAACGCTACTCCA 3')	405
	wsp 328F (5' CCAGCAGATACTATTGCG 3') wsp 691R (5' AAAAATTAAACGCTACTCCA 3')	379
Group B	wsp 81F (5' TGGTCCAATAAGTGATGAAGAAAC 3') wsp 522R (5' ACCAGCTTTTGCTTGATA 3')	442
	wsp 183F (5' AAGGAACCGAAGTTCATG 3') wsp 691R (5' AAAAATTAAACGCTACTCCA 3')	501

(Baldini *et al*., 2014; Dyab *et al*., 2016; Shaw *et al*., 2016; Nugapola *et al*., 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 CHEMICALS AND REAGENTS

3.0 The following chemicals and reagents which are of analytical grade were products of Sigma Aldrich (St Louis , Missouri United States of America)

- Silica
- 10x Standard Taq buffer with 25mM $MgCl_2$
- dNTPs
- Taq Polymerase
- DNeasy water
- Primers (forward and Reverse)
- 50x TAE (stock solution)
- Agarose
- Ethidium bromide
- Wide range DNA ladder A401A
- Loading dye

Few others (listed below) are products of Zymo research (Irvine, United States of America).

- DNA extraction kit (quick DNA^{TM} miniprep plus kit
- Mosquito DNA extraction protocol kit

3.1.1 EQUIPTMENT

- Micropipette, P-20 , P- 200, P- 1000 (Model Eppendorf)
- Microcentrifuge (Model Eppendorf)
- Vortex mixer SA8 variable speed Mfr.No.0472907
- Gradient mastercycler (nexus) Eppendorf AG 22331, Hamburg
- Gel documentation. Chem Doc™ XRS, Bio-Rad
- Oven (LAT1000AAE)
- Microscope (Olympus Hamburg, Germany)
- Nanodrop Spectrophotometer (Denovix DS-11+)
- Chest Freezer, HTF 319H Haier, Thermocool 319LTR
- Shaking water bath Thermo Scientific™ precision SWB27 TSSWB27, Precision™
- Centrifuge VWR®, Compact star CS4
- Electrophoresis System, MultiSUB®

3.2 STUDY AREA

The Gambia is a West African country that lies between latitude 13^0 and 14^0 and longitude 13^0 and 17^0. The country is almost completely surrounded by Senegal except on the western side where it bordered with the Atlantic Ocean. The River Gambia which takes its source from Futa Jallon Highlands in Guinea and empties it into the Atlantic Ocean, divides The Gambia into two banks (North and South). The country has an area of 10,689 square kilometers with a population of 1,882,450 based on 2013 census. The climate is typically sub-sahelian. It has two seasons; the rainy season which starts at end of May and ends in mid-October and the dry season (which is the longest of the two) starts in late October and ends in early May. The Gambia is divided into eight Local Government Areas namely: Banjul, Kanifing , Brikama, Mansa konko, Kerewan, Kuntaur, Janjanbureh and Basse. For the purpose of this research , the former divisons as shown in Fig 3.1 was used; Banjul, Western, North Bank, Lower River, Central River and Upper River division.

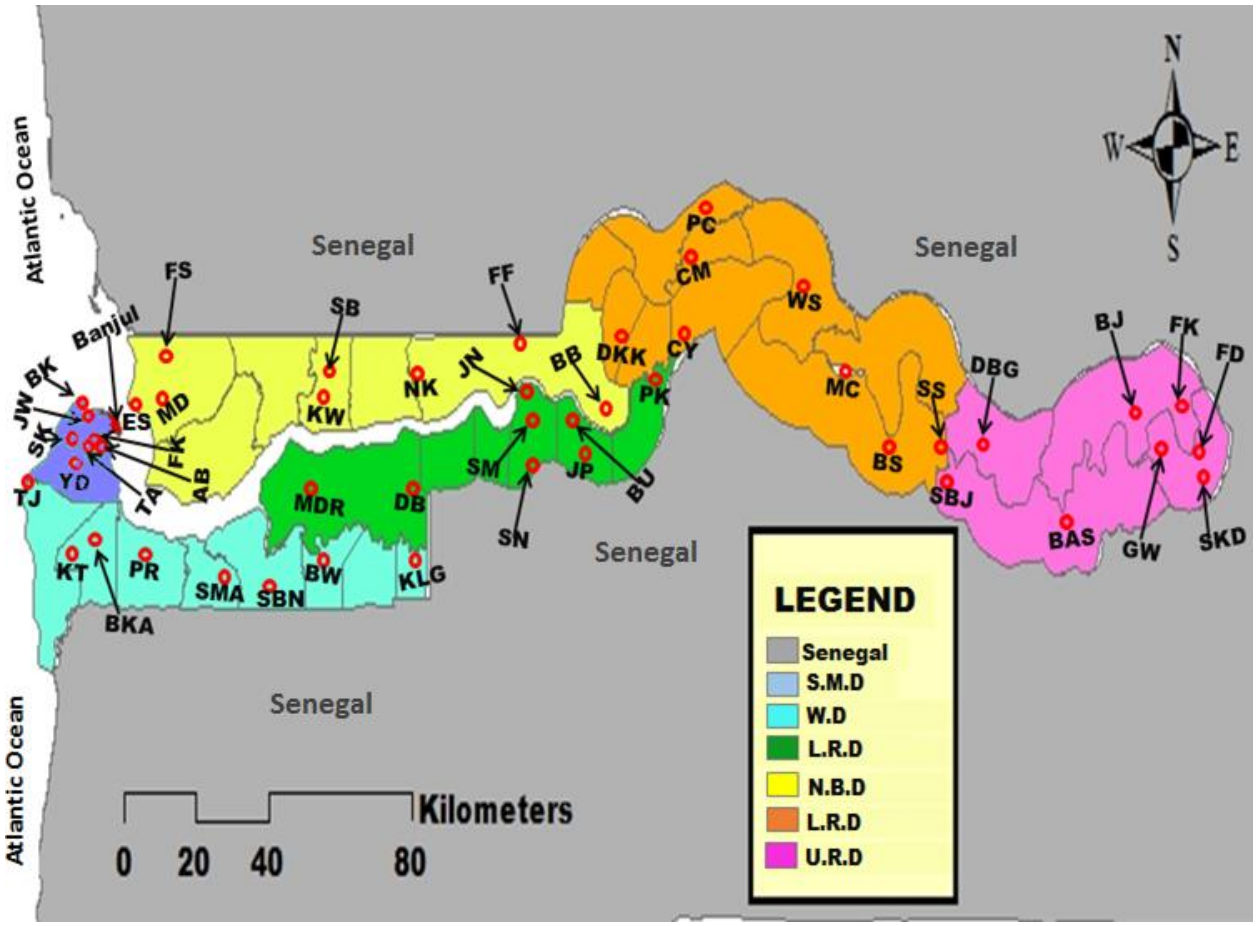


Fig 3.1: Map of the Gambia indicating the six divisions.

KEY

SMD	WD	NBD	LRD	CRD
FK = Fagi Kunda	TJ = Tanji	ES = Essau	SM = Soma	MC= Macaty
TA = Tallinding	PR = Pirang	KW= Kerewan	MDR = Manduar	CY - Choya
SK = Sukuta	BW = Bwiam	NK= No Kunda	JN = Jinoi	DKK=Dankunku
BK = Bakau	SMA = Somita	FS = Fass	PK = Pakaliba	CM= Chamen
JW = Jeswang	SBN = Sibonor	SB = Saaba	SN = Senoba	PC= Panchang
AB = Abuko	KT = Kiti	FF = Farafenni	DB = Dumbuto	WS= Wassu
YD = Yundum	BKA = Brikama	BB = Bambali	BU = Buiba	SS = Sare Sofi
Banjul = Banjul	KLG = Kanglagi	MD = Madina	JP = Japineh	BS= Bansang

URR

BAS= Basse SKD = Song Kunda FT= Fatoto GW= Garawol BJ= Baja DBG = Diabugu
SBJ = Sare Bojo FD = Foday Kunda

3.3 SAMPLE SIZE

The sample size was determined by using the formula $N = \frac{Z^2pq}{d^2}$ as described by Thrusfield (2007) based on 95% confidence interval and prevalence of 50%.

From the formula:

N is the sample size of pools of mosquito (to be calculated)

Z = 1.96 for 95% confidence level

p= 0.5 (Prevalence)

q= Complementary probability (1-0.5)

d= 0.05

$N = \frac{1.96x1.96x0.5x0.5}{0.05x0.05}$

$N = \frac{0.9604}{0.0025}$

N = 384 pools of mosquitoes only

3.4 LIMITATIONS

This study is limited to anthropophilic adult mosquitoes only.

3.5 SAMPLING TECHNIQUES

Multi stage sampling technique was employed. For each division, 8 settlements were selected through balloting, and for each settlement, 8 compounds were selected through balloting. The compounds selected are the sampling points/pools. This equates to 64 sampling points/pools for each division giving a total of 384 sampling points/pools for the entire country.

3.6 STUDY UNITS

Residential houses and the surroundings

3.7 SAMPLING SITES

Mosquito samples were collected from all the divisions in The Gambia.

Table 3.1 . Shows the sampling sites for each division.

DIVISIONS	SAMPLING SITES
SMD	Fagikunda Banjul Tallinding Sukuta Bakau Jeswang Abuko Yundum
WD	Tanji Pirang Bwiam Somita Sibanor Kiti Brikama kanglagi
NBD	Essau Kerewan No kunda Fass Saaba Farafennni Bambali medina
LRD	Soma Manduarr Jinoi Pakaliba Senoba Dumbuto Buiba Japineh
CRD	Macaty Choya Dankunku Chamen Panchang Wassu Sare Sofi Bansang
URD	Basse Song kunda Fatoto Garawol Baja Diabuqu Sare bojo Foday kunda

SMD St Mary's Division

WD Western Division

NBD North Bank Division

LRD Lower River Division

CRD Central River Division

URD Upper River Division

.

3.8 COLLECTION OF MOSQUITO SAMPLES

3.8.1 Collection of mosquitoes using adaptation of the G' rumba container

G' rumba is a name of a container in Jola language made of clay. Jola is a tribe in the Senegambia region and some parts of Guinea Bissau deeply rooted in herbal medicine. The container is mostly used by herbalists in the preparation and storage of concoctions. It has been observed that while in storage, a scourge of mosquitoes seeks shelter and a source of water for drinking in such containers. The container could also be a breeding ground if kept in that position for weeks. It is from this concept, this study developed a trap for collecting mosquitoes for purpose of this research. The materials used here are buckets and transparent piece of clothes sawed into sacs with a control valve made of rope. In the setup, water is filled into the buckets to one quarter full. Small branches with leaves of shrubs were then put inside to create a bushy environment that can be a temporary home for mosquitoes and other flies within that environment. The harvesting processes involved carefully capping the mouth of the bucket with the mouth of the designed sac such that when the bucket is shaken the mosquitoes will run into the sacs. The sac can then be removed carefully and the valve closes to prevent any trapped mosquito from escaping. The activities of mosquitoes are time bound. Some genera are active during the night while others are active in some parts of the day. At around midday, mosquitoes are generally not active. During such periods, they could be found hanging on walls in dark areas or any other surfaces nearby. In this method of collection, the traps were set to provide such resting platforms for the mosquitoes during such inactive periods of their time. The traps were positioned in baths rooms, verandahs, sitting rooms and other immediate environments.

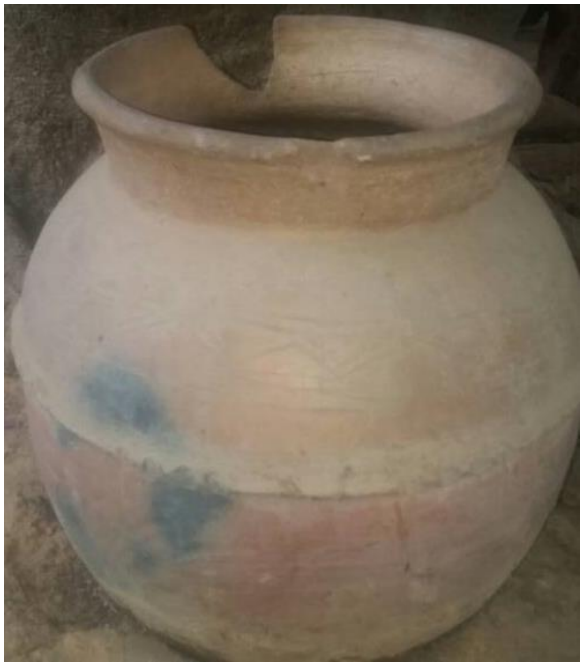


Plate 2.10: G’ruumba



Plate 3.2 G’ruumba Trap (Modified mosquito trap)

The compound owners and the community were sensitized on the whole operation. The selected area for the traps were cleared of any other material that could provide a resting platform for the mosquitoes. The traps were laid from 8am in the mornings to 2pm the next day (a period of 30hrs). Harvests were made at 2pm as described above. The live mosquitoes were left in the sacs for 48hrs to weaken and then transferred into tubes containing silica in sachets (for preservation) and labeled to indicate division, pool number and date of harvest. Once harvested, the small branches with leaves were discarded and the water poured to avoid the setup being used by mosquitoes for breeding. The mosquitoes were then transported to Department of Biochemistry Ahmadu Bello University, Zaria Kaduna State, Nigeria for laboratory analysis.

3.9 IDENTIFICATION OF THE MOSQUITOES

The mosquitoes were identified per pool into genera level according to Stone *et al*. (1959), using microscopy at the Department of Veterinary Parasitology and Entomology Lab, Ahmadu Bello.University, Zaria. The main taxonomic features used were : body colour, spotted and unspotted wings, length of palps in comparison with their proboscis and presence of black strips on body and legs . The number of each genera of mosquito for each pool were established and placed in tubes and labeled. The sex of the mosquitoes were also determined for every pool based on the presence of bushy feathers around the antenna. For Improving the sensitivity and cost effectiveness in the DNA analysis by PCR, each genera of mosquitoes in the sampling points/pools of each settlement/ cluster were pooled together. This translates into 48 clusters of *Anopheles* genera mosquitoes, 48 clusters of *Culex* genera mosquitoes and 24 clusters of *Aedes* genera of mosquitoes. DNA was extracted from 120 clusters of mosquitoes.

3.10 IDENTIFICATION OF *WOLBACHIA* AND MICROFILARIAE

3.10.1 DNA extraction

Dry mosquito samples by cluster were homogenized by grinding into powder in eppendorf tubes (one for each cluster) using fresh micropestle for each cluster. 100µl of distilled water was added to each of the samples with less than 5 mosquitoes while 200µl of distilled water was added to samples with more than 5 mosquitoes. The samples were briefly vortexed to distribute the particles evenly. Quick-DNA ^{TM} miniprep plus kit (Zymo) protocol was used as follows:

i. 95µl of solid tissue buffer (blue) and 10µl of proteinase k were added.

ii. The mixture was vortexed for 1 minute and incubated at 55^0C for 1hr 30minutes.

iii. The mixture was centrifuged at 12,000 xg for a period of 1 minute at 4^0 C

iv. 200µl of the supernatant was transferred into a clean eppendorf tube by pipetting.

v. 400µl of genomic binding buffer was added to the 200µl of the supernatant and vortexed for 1 minute.

vi. The mixture was then transferred into zymo-spin 11-XL column. The column was placed on a collection tube.

vii. The mixture was centrifuged at 12,000 xg for a period of 1 minute at 4^0C.

viii. The collection tube with the flow through were discarded

ix. 400µl of DNA pre-wash buffer was added to the spin column and centrifuged at 12,000xg for a period of 1 minute at 4^0C

x. The collection tube with the flow through were discarded

xi. The column was given a second wash with 700µl of g-DNA wash buffer and centrifuged at 12,000 xg for a period of 1 minute at 4^0C.

xii. The collection tube with the flow through were discarded

xiii. The column was given a last bath by adding 200µl of g-DNA wash buffer and centrifuged at 12,000 xg for a period of 1minute at 4^0C.

xiv. The collection tube and the flow through were discarded

xv. Finally to elute the DNA, 50µl DNA elution buffer was added to the column and centrifuged at 12,000 xg for a period of 1 minute at 4^0C.

xvi. The column was discarded and the flow through which supposed to contain the DNA was appropriately labeled and stored at -20^0C until needed for quantification

3.10.2 DNA quantification using Nanodrop Spectrophotometer

The DNA samples were removed from the freezer, thawed and briefly vortexed. The lower and the upper pedestals of the nanodrop Spectrophotometer (Denovix DS- 11+) were cleaned and blanked with 2µl of deionized water. The samples were then run one after the other with intermittent cleaning of the lower and the upper pedestals with tissue paper after every sample run. The concentration of the DNA and the absorbance of 260/280 ratio (for purity) were recorded.

3.10.3 Identification of Wolbachia DNA using 16SrDNA and wsp gene

3.10.3.1 Detection using 16S rDNA

To determine the presence of *Wolbachia* DNA , PCR technique was employed using 16S Wolb F and 16S Wolb R (forward and reverse primers) specific primers: 16S rDNA (W-Specf 5'-CATACCTATTCGAAGGGATAG-3', W-Specr 5'-AGCTTCGAGTGAAACCAATTC-3') (Baldini *et al.*,2014) The amplification were carried out with a Gradient Thermo Cycler according to Plichart *et al.*, (2005). Average DNA concentration from extraction was below

50ng/µl, therefore the volume of DNA template used was increased from 2ng/µl to 4ng/µl. The reaction mixture for each of the 120 cluster sample consisted of 4µl of DNA template, 2.5µl of 10X buffer, 2.5µl of 2.5mM (each) dNTPs, 0.35µl of 20µM each of forward and reverse primers (16SWolbF/16SWolbR), 0.25 µl of 5U/µl Taq DNA polymerase and a volume of 15.05µl DNAse free water to make a final reaction volume of 25 µl. The amplification reaction protocol used was as follows:-

- initially of 2 min at 95ºC
- 40 cycles of 30sec at 95ºC,
- 30 sec at 55ºC
- 30 sec at 72ºC,
- and a final extension of 3 min at 72ºC.
- 4^0C (for cooling)

These conditions are expected to yield fragments of ~438bp (Baldini *et al*., 2014 and Dyab *et al* 2016; Shaw *et al*.,2016)

The amplicons where kept at -20^0C until needed for gel electrophoresis

3.10.3.2 Classification of *Wolbachia* into Supergroups

Wolbachia in mosquito population are of two supergroup: Supergroup A or B. Wsp group specific primers were used to detect *Wolbachia* in the three genera of mosquitoes, although wsp is reported of being insensitive in detecting *Wolbachia* in anopheles mosquitoes (Kittayapong *et al*.,2000; Wiwatanaratanaburt, 2013; Wakala *et al*.2017).

i) Detection of *Wolbachia* Supergroup A using wsp136F and wsp691R

All positive samples from 16SrDNA screening were also subjected to wsp136F/wsp691R screening. The primer sequence are wsp136F 5' TGAAATTTTACCTCTTTTC 3' and wsp691R 5' AAAAATTAAACGCTACTCCA 3'. These primers are specific to *Wolbachia* supergroup A. PCR amplification were carried out for all the positive samples for 16SrDNA. A total reaction volume for a single reaction used was 25µl. The mix contained 4µl of DNA template, 2.5µl of 10x reaction buffer, 2,5µl of dNTPs mix, 0.35µl each of forward (wsp136f) and reverse (wsp691r) primer, 0.127µl of 5U/µl Taq polymerase and 15.175µl of DNAse free water. The PCR reaction conditions were as follows:

- 1 cycle of 1 min at 94^{o}C
- 35 cycles of 15sec at 94^{o}C
- 30 sec at 55^{o}C
- 1 min at 72^{o}C
- 7 min at 72^{o}C
- 4^{o}C cooling

The expected amplicons size is ~ 556bp (Nugapola *et al.*, 2017).

ii) Detection of *Wolbachia* Supergroup B using wsp81F and wsp522R

All positive samples for *Wolbachia* 16srDNA were subjected to wsp81F/wsp522R screening. The primer sequence are: Forward primer wsp81F 5' TGGTCCAATAAGTGATGAAGAAC 3' and a reverse primer wsp522R 5' ACCAGCTTTTGCTTGATA 3' .These primers are specific to *Wolbachia* supergroup B. PCR amplification were carried out for all the positive samples for 16srDNA. A total reaction volume for a single reaction used was 25µl. The mixture contained

4µl of DNA template, 2.5µl of 10x reaction buffer, 2,5µl of dNTPs mix, 0.35µl each of forward(wsp136f) and reverse (wsp691r) primer, 0.127µl of 5U/µl Taq polymerase and 15.175µl of DNAse free water. The PCR reaction conditions were as follows:

- 1 cycle of 1 min at 94^{o}C
- 35 cycles of 15sec at 94^{o}C
- 30 sec of 55^{o}C
- 1 min at 72^{o}C
- 7 min at 72^{o}C
- 4^{o}C for cooling

The expected band size is ~ 442bp (Nugapola *et al.*, 2017).

3.10.4 Identification of Microfilariae Species

3.10.4.1 *Wuchereria bancrofti*

The sspI gene is a repeat sequence which is a signature for *W. bancrofti*. PCR with specific primers for the SspI gene were used to detect the presence of *W. bancrofti* in the sample .The forward primer for the SspI gene of *W. bancrofti* was sspI F 5'-CGT GAT GGC ATC AAA GTA GGG-3', and the reverse primer for the SspI gene was SspIR 5'-CCC TCA CTT ACC ATA AGA CAAC-3'. The PCR amplification for 48 cluster samples were carried out in a gradient thermal cycler. A total reaction volume of a single reaction of 25µl, contained 4µl of DNA template, 2.5µl of 10x reaction buffer, 2,5µl of dNTPs mix, 0.35µl each of forward and reverse primer, 0.127µl of 5U/µl Taq polymerase and 15.175µl of DNAse free water. The PCR reaction conditions were as follows:.

- 95^{0}C for 5 min
- 38 cycles of 1min at 94^{0}C

- 1 min at 56^0C
- 1 min at 72^0C
- 10 min at 72^0C.
- 4^{o}C cooling

These conditions would yield a PCR product of 188bp (Siridewa et al., 1996).

3.10.4.2 *Brugia malayi*

The hhaI gene in *Brugia malayi* is a tandem repeat sequence of about 320bp. The hhaI gene is the target in the detection of *Brugia malayi*. The forward primer used was hhaI F 5'-GCG CAT AAA TTT ATC AGC-3' and the reverse primer was hhaI R 5'- GCG CAA AAC TTA ATT ACA AAA GC-3'. The amplification of the gene was carried out using a Gradient Thermal cycler. A total reaction volume of each of the 73 cluster samples used was 25µl. This contained 2µl of DNA sample, 2.5µl of 10x reaction buffer, 2.5µl of dNTPs mix, 0.35µl of each of forward and reverse primers, 0.125µl of 5U/µl of Taq polymerase and 15.175µl of DNase free water.

The reaction protocol was as follows:

- 95^0C for 5 min
- 38 cycles of 30 sec at 95^0C
- 30sec at 56^0C
- 30 sec at 72^0C
- 5 min at 72^0C
- 4^{o}C cooling

These conditions would yield a PCR product of 320bp (Vasuki et al.,2001; Noordin., 2010).

3.10.5 Gel electrophoresis

Procedure:

3.10.5.1 Preparation of Ethylenediamine tetraacetic acid (EDTA)

A hundred mililitre (100ml) of 0.5M EDTA (molecular weight 292.24g/mol) was prepared by dissolving 18.61g of disodiumethylenediametetraacetate in 100 ml deionized water. The pH was adjusted by adding drops of 5M sodium hydroxide solution, using a Microprocessor pH meter to a pH of 8.0

3.10.5.2 Preparation of Tris-acetate- EDTA (TAE) buffer

A stock solution of 50x TAE buffer was prepared according to manufacturer's instructions. 242g of Trisbase (mw 121) was dissolved in 600 ml of deionized water by thorough shaking. 57.1ml of glacial acetic acid and 100ml of 0.5M EDTA (pH 8.0) were added and stired. The volume of the solution was adjusted to 1000ml. A working solution of 1x of 500ml was then prepared by adding 10 ml of 50x into 490 ml of deionized water.

3.10.5.3 Preparation of the Gel

One grame of agarose was dissolved in 100 ml deionized water to make 1% agarose solution. The solution was boiled in a microwave. The gel was stained by adding 6µl Ethidium bromide (EtBr). The gel was then cast while the combs were in position. After the gel solidified, carefully the combs were carefully removed and the gel ready to be used.

3.10.5.4 Loading the sample

The gel containing 20 wells of which two DNA molecular weight marker (M) were loaded. Water was used as negative control (N) at the far right of each gel. The remaining wells were loaded with 10µl (8µl of amplicons and 2µl of the loading dye) of the sample. Enough 1X TAE buffer was then poured into the tank to submerge the gel. A voltage of 109 was used to run the gel for 30minutes. After 30 minutes of run, the gel was viewed and documented using a BioRad Gel Documentation System..

3.11 STATISTICAL ANALYSIS

The association between Wolbachia and different genera of mosquitoes were analysed using bivariate fit of quadratic regression of Statistical Analysis System (SAS 9.4). Relationship between variables were determined using correlation analysis. Charts were fitted in Microsoft Excel. Significant association was set at $p < 0.05$.

CHAPTER FOUR

4.0 RESULTS

4.1 PURITY ASSESSMENT OF THE EXTRACTED DNA

The purity of the extracted DNA determined by Nanodrop Spectrophotometer (Denovix Ds – 11+) expressed as ratio of absorbance at 260/280 were with the exception of few samples, in the range of 1.2 – 1.8.

4.2 Distribution of Anthropophilic Mosquito Genera In The Gambia.

A total of 2003 anthropophilic mosquitoes from six divisions of The Gambia were classified into genera by microscopy method (magnification X10). Three genera of anthropophilic mosquitoes were identified as *Anopheles*, *Culex* and *Aedes*. *Mansonia* genera of mosquito was not detected in the total anthropophilic mosquitoes collected (Table 4.1). A total of 65.0% of the anthropophilic mosquitoes in the Gambia are *Anopheles* genera, 31.9% are *Culex* genera, while 3.1% belong to *Aedes* genera. None of the anthropophilic mosquitoes belongs to the *Mansonia* mosquito genera. The distribution pattern of the genera was similar across the six divisions.

Figure 4.2 shows the pooled samples of anthropophilic mosquitoes in different divisions in The Gambia. There was significant differences ($P<0.05$) among the mosquito genera across the division. SMD and CRD had the highest population of mosquitoes which were significantly ($P<0.05$) different from WD, URD, NBD and LRD.

Table 4.1 population of anthropophilic mosquitoes by genera in dufferent divisions.

		Frequency (%)						
Division	**A**	**Anopheles**	**C**	**Culex**	**Ae**	**Aedes**	**M**	**Mansoni**
SMD (n= 411)	216	52.6	165	40.1	30	7.3	0	0
WD (n= 354)	265	74.7	84	23.9	5	1.4	0	0
NBD (n= 294)	207	70.4	81	27.6	6	2.0	0	0
LRD (n= 254)	176	69.3	68	26.8	10	3.9	0	0
CRD (n= 390)	255	65.4	129	33.1	6	1.5	0	0
URD (n= 300)	180	60.0	114	38.0	5	2.0	0	0
Total 2003	1299	64.9	641	32.0	62	3.1	0	0

Key : SMD = St marys Division
WD = Western Division
NBD = North Bank Division
LRD = Lower River division
CRD = Central River division
URD = Upper River Division

A = number of *Anopheles* mosquitoes
C = number of *Culex* mosquitoes
Ae = number of *Aedes* mosquitoes
M = number of *Mansonia* mosquitoes

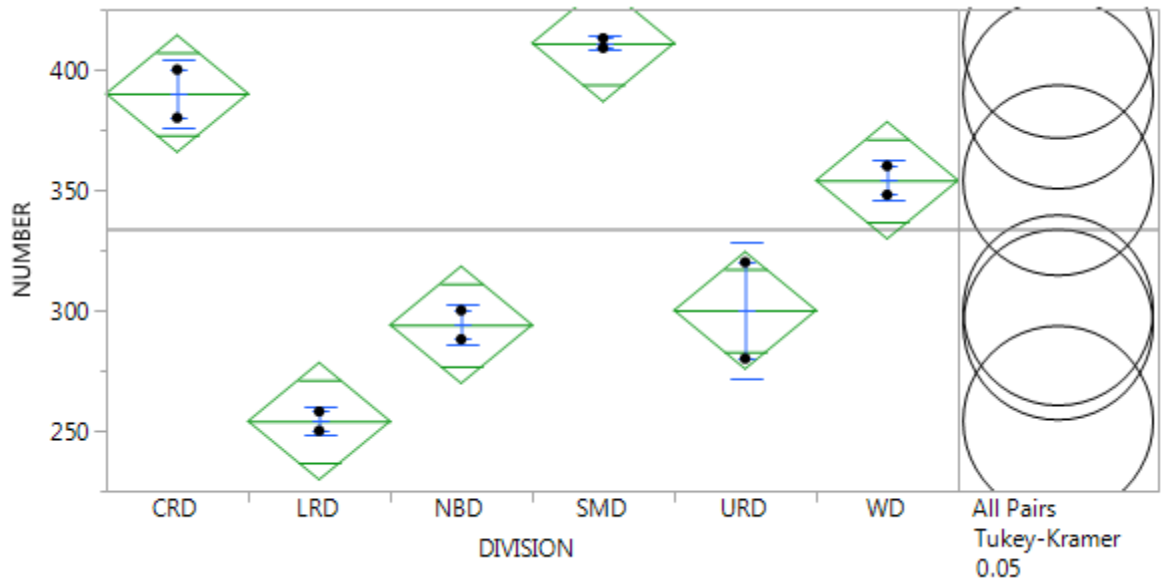


Figure 4.2 Pooled samples of anthropophilic mosquitoes in different divisions in the Gambia.

Key:

CRD = Central River Division
LRD = Lower River Division
NBD = North Bank Division
SMD = St Marys Division
URD = Upper River Division

4.3 Sexual Dimorphism of Anthropophilic Mosquito Genera

Sexual dimorphism of 2003 anthropophilic mosquito from the six divisions in The Gambia were determined by microscopic technique. Of the 2003 anthropophilic mosquitoes sampled, 82.1% were female while 13.0% were males. (Table 4.2) There is a significant different ($p < 0.05$) in sexual dimorphism among anthropophilic mosquitoes in The Gambia.

Table 4.2 Sexual dimorphism of anthropophilic mosquitoes in different divisions in The Gambia

		Frequency (%)		
Division	**No. Female**	**% Female**	**No. Male**	**%Male**
SMD (n= 411)	386	93.9	25	6.1
WD (n= 354)	299	84.4	55	15.5
NBD (n= 294)	232	78.9	64	21.8
LRD (n= 254)	206	81.1	48	18.9
CRD (n= 390)	352	90,3	38	9.7
URD (n= 300)	269	89.7	31	10.3
TOTAL 2003	**1744**	**87.0**	**261**	**13.0**

Key : SMD = St Marys Division

WD = Western Division

NBD = North Bank Division

LRD = Lower River Division

CRD = Central River Division

URD = Upper River Division

4.4 DETECTION OF WOLBACHIA AND CLASSIFICATION INTO SUPERGROUP.

Results of the DNA analysis from 120 clusters of mosquitoes for presence of *Wolbachia* and supergroup using 16SrDNA and wsp primers are presented in Plate 4.1- 4.3 and Table 4.3.The results indicated a 34.17% incidence (Table 4.3) of *Wolbachia* in anthropophilic mosquitoes in The Gambia. All the three genera of mosquitoes were infected with *Wolbachia* (Plate 4.1;4.2). A higher proportion of the *Anopheles* harbored *Wolbachia* (50%), while *Aedes* had a significantly lower rate. The *Wolbachia* detected in *Culex* and *Aedes* belong to Supergroup B (Plate 4.4). Though *Wolbachia* was detected in Anopheles genera using 16SrDNA, the supergroup could not be determined using wsp primers. This was due to the insensitivity of wsp primers in detecting *Wolbachia* in *Anopheles*.

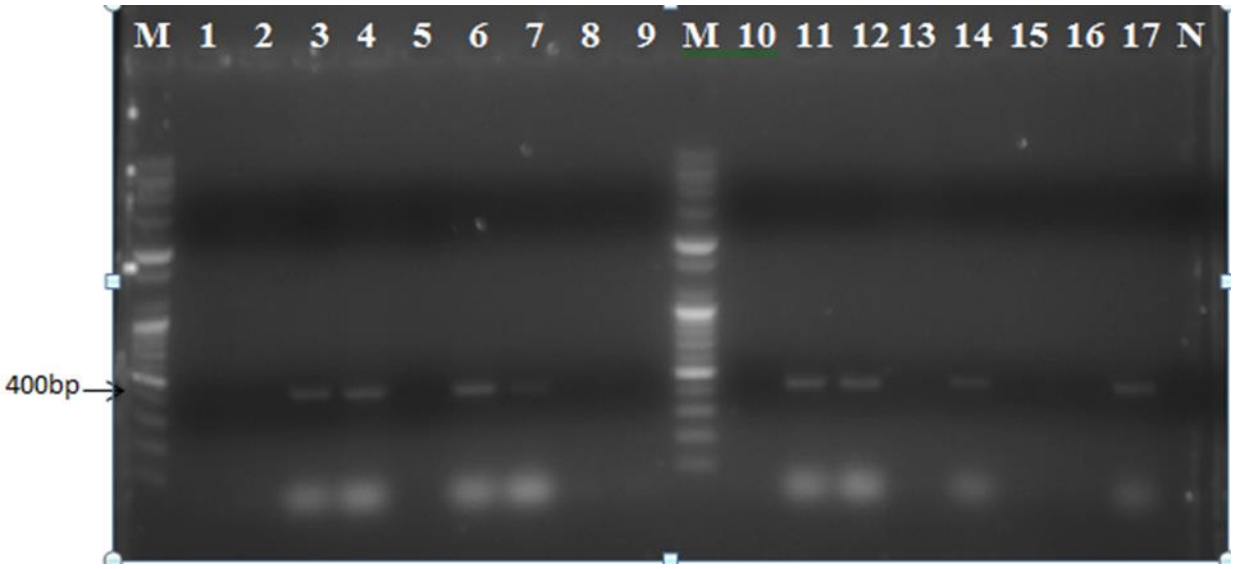


Plate 4.I: PCR amplification of the 16SrDNA (438bp) of *Wolbachia* in anthropophilic mosquitoes.

M - Molecular weight marker

N- Negative control.

Lanes 3,4,6 and 7 +ve Anopheles samples.

Lanes 11, 12and 14 +ve Culex samples

Lane17 +ve Aedes samples

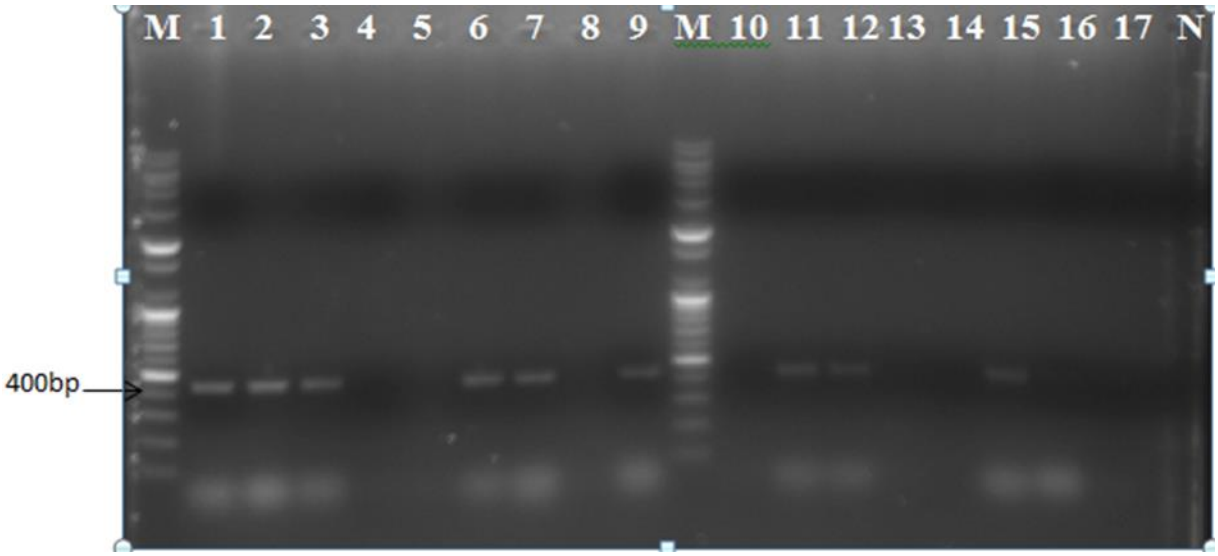


Plate II: .PCR amplification of the 16SrDNA (438bp) of *Wolbachia* in anthropophilic mosquitoes

M - Molecular weight markers.

N - negative control.

Lanes 1,2,3,6 and 7 +ve Anopheles samples.

Lanes 9,11,12 and 15 +ve Culex samples

Table 4.3 Pooled samples of *Wolbachia* incidence in anthropophilic mosquito genera

Mosquito				**Frequency**	
Genera	**Cluster**	**No. positive**	**No. negative**	**% positive**	**%negative**
Anopheles	48	24	24	50	50
Culex	48	15	33	31.25	68.75
Aedes	24	2	22	8.3	91.7
	120	41	79	34.17	65.83

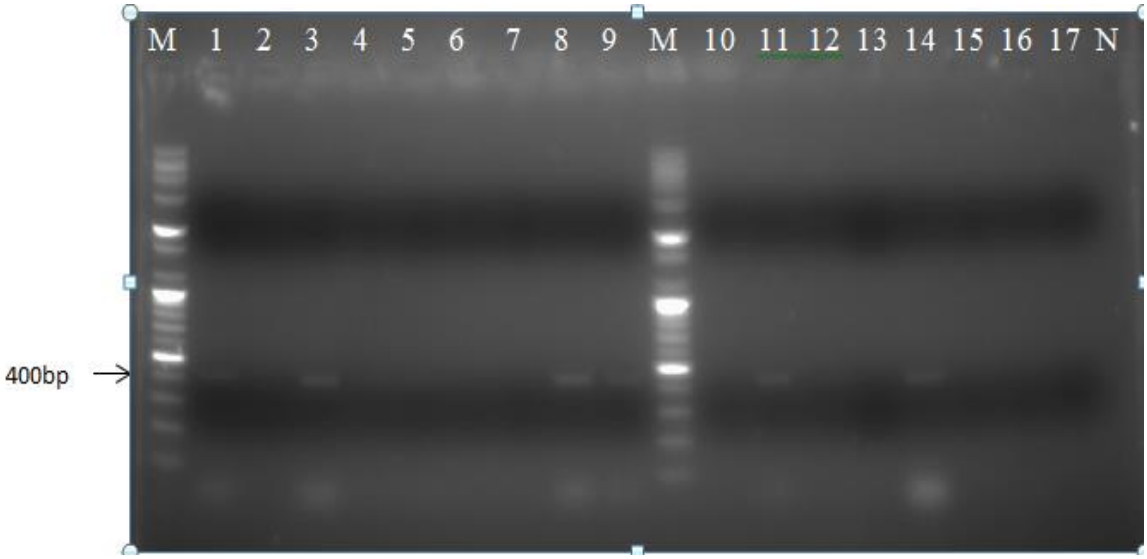


Plate III. PCR amplification of the wsp (442bp) for *Wolbachia* Supergroup B in anthropophilic mosquitoes.

M - Molecular weight marker

N - Negative control.

Lanes 1, 3, 6, 7, 8, 9,11, 12 +ve wolbachia supergroup B in Culex samples

Lane 14 +ve wolbachia supergroup B in Aedes samples

Table 4.4 Distribution of *Wolbachia* supergroups among anthropophilic mosquito genera in the Gambia.

Mosquito			**% Frequency**	
Genera	**No. Cluster**	**No. positive**	**WolbA**	**WolbB**
Anopheles	48	0	0	0
Culex	48	15	0	31.3
Aedes	24	2	0	8.3
	120	17	0.0	14.17

4.5 *Wolbachia* – Mosquito Association

The association of *Wolbachia* and anthropophilic mosquitoes genera in The Gambia was analysed using bivariate fit of quadratic regression of statistical analysis system (SAS 9.4). The analysis in Table 4.5 shows that *Wolbachia* has high and significant association ($P<0.05$) with *Culex*. The association between *Wolbachia* and *Culex* was strong and positive ($r=0.79$) with cofficient of determination of 63%. Association of both *Anopheles* and *Aedes* with *Wolbachia* is not significant ($P>0.05$), though the correlation was high between wolbachia and *Anopheles* ($r=0.6$), the correlation between *Wolbachia* and *Aedes* is low (0.61).

Table 4.5: Bivariate fit of anthropophilic mosquito genera and *Wolbachia*

Genera	RMSE	R^2	r	Pvalue
Culex	36.4	0.63	0.79	0.05*
Anopheles	84.35	0.36	0.60	0.29^{ns}
Aedes	7.63	0.37	0.61	0.25

RMSE – Root mean square error

R^2 - Coefficient of determination

r - Correlation

p- probability of regression coefficient I

4.6 Screening for the presence of *W.Bancrofti* and *M. Malayi* DNA in Anthropophilic Mosquitoes.

Results of the screening of the DNA of the mosquitoes for filarial nematode using ssp1 and hhaI gene primers (Plate 4.4) indicated that there were no nematode in the sampled mosquitoes . Both *W.bancrofti* and *B.malayi* were absent in the mosquitoes in the six divisions of The Gambia.

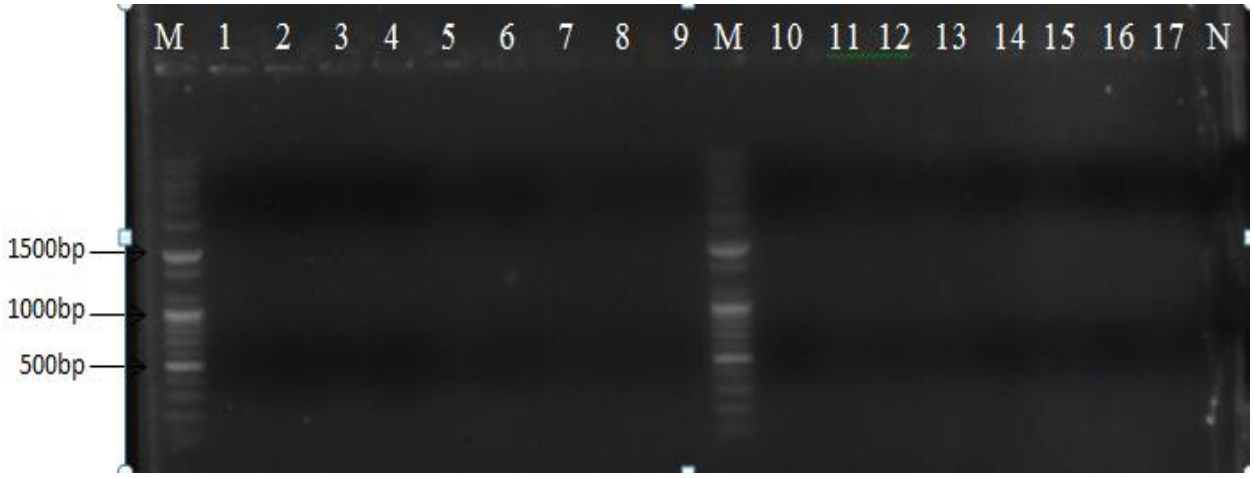


Plate IV: PCR amplification of the sspI (188bp) and hhaI (320bp) of *W.bancrofti* and *B. Malayi* in anthropophilic mosquitoes.

M =Molecular weight marker

N - negative control

Lanes 1,2,3,4,5,6,7,8,9 -ve *Anopheles* samples

Lanes 10,11,12,13 and 14 -ve *Culex* samples

Lanes 15, 16 and 17 –ve *Aedes* samples

CHAPTER FIVE

5.0 DISCUSSION

Various genera of mosquitoes in the Gambia are distributed across all the divisions of the country, with the exception of *Mansonia* genera which was not detected among the anthropophilic family of mosquitoes sampled. Availability of fresh , clean or turbid water plays an important role in the distribution of mosquitoes. Fresh water body from rivers or ponds constitutes the highest environments for mosquito breeding sites. Although SMD and WD lacked fresh river waters, however, urbanization has played a major role in providing mosquito breeding grounds through numerous sewages and waste water bodies produced daily by human activities. In CRD and URD, the high population could be attributed to the existence of fresh water bodies from the river which could provide a good breeding grounds for the mosquitoes. NBD and LRD registered the lowest in anthropophilic mosquito population compare to the other divisions. These two divisions are characterized by sparsely distributed populations produces less sewages and waste water bodies couple with salty river waters reduces the chances of mosquito breeding compared to other divisions. The mosquito population in these two divisions are thus hampered by lack of adequate fresh water bodies all year round. The high proportion of *Anopheles* genera among mosquitoes in The Gambia is not unexpected. *Anopheles* is not restricted to clearly defined habitats for breeding. The genera has the potentials to breed in all open water bodies (Laporta *et al*., 2011). The lower proportion of *Anopheles* in SMD when compared to other divisions, could be attributed to the fact that SMD is the most highly dense in human population with the highest industrial activities. SMD has also been designated as the most polluted divisions. *Anopheles* mosquitoes generally prefer unpolluted water bodies for breeding (Geissbuhler, 2008; Laporta *et al*., 2011). It has been reported that pollution as a result of

urbanization has eliminated certain species of *Anopheles* from urban centers (Geissbuhler, 2008; Laporta *et al*., 2011). The least populated among the three existing genera in anthropophilic mosquitoes is the *Aedes* genera. *Aedes* genera generally breeds in artificial containers such as pots, tyres , open barrels containing clean water mainly of rain or natural containers of rain water such as holes in trees (Paupy *et al*., 2009; Nazri, *et al* .,2013 ; D ejene *et al*., 2015). The increased awareness in the fight against mosquito borne diseases which have resulted in the cleaning of the environment, by collecting discarded pots, tyres or any container which could hold water for mosquito breeding and the decline in amount of rain water received in recent times might have contributed to the low occurrence of Aedes mosquito genera in The Gambia.

Difference in sexual dimorphism among anthropophilic mosquitoes in The Gambia is significantly higher in SMD, WD, CRD and URD where *Wolbachia* infection was evident in this study. The relationship between these two factors (mosquito sex and Wolbachia) has been explained by several researchers., *Wolbachia* induces cytoplasmic incompatibility in arthropods and distort the sex ratio (Bordenstein et al., 2001; Charlat *et al*., 2006; Telschow *et al*., 2007). Through cytoplasmic incompatibility *Wolbachia* modifies the spermatozoa such that male mosquitoes dies early in embryogenesis and also *Wolbachia* was also reported to have the potentials to feminize male mosquitoes during early embryogenesis. The significant differences in sexual dimorphism observed in this study may be attributed to the presence of *Wolbachia* in anthropophilic mosquito populations.

The three genera of mosquitoes; *Anopheles, Culex* and *Aedes* among anthropophilic mosquitoes in The Gambia are infected with Wolbachia. The incidence of infection is greater in *Anopheles*

than Culex and Aedes. Although the mosquito – *Wolbachia* association is significant only in Culex, only the supergroup B of *Wolbachia* was identified in *Culex* and *Aedes* anthropophilic mosquitoes in The Gambia. The supergroup in *Anopheles* could not be detected by wsp primers in this study as also reported by kittayapong *et al*., (2000); Wiwatanaratanaburt, (2013); Nalaka *et al*., (2017). The insensitivity of wsp primers in detecting *Wolbachia* in *Anopheles* mosquitoes could be attributed to some form of mutation in the surface protein gene in *Wolbachia*. The inability of wsp primers in detecting *Wolbachia* has previously led to the conclusion that *Anopheles* mosquitoes are not naturally infected with *Wolbachia* (Grant *et al*.,2011). This conclusion spurred scientists in the World to attempt introducing *Wolbachia* artificially into natural population of *Anopheles* (Grant *et al.*, 2011). However, screening with 16srDNA has now revealed that *Anopheles* mosquito can also harbor *Wolbachia*. The supergroup of the *Wolbachia* in *Anopheles* will be better determined by sequencing.

None of the 120 clusters of anthropophilic mosquitoes (comprising 2003 mosquitoes) was positive for *W.bancrofti* and *B.malayi*, suggesting absence transmission of lymphatic filariasis in The Gambia at the sampling time. This finding is in agreement with the conclusion of Rebello *et a*l., (2015) who stated that there is no longer active filarial transmission in the country as at the time of their study. It is probable that the high incidence of *Wolbachia* infection in anthropophilic mosquitoes could have contributed to this. This assumption is further strengthened by the very high dimorphic difference among the mosquito sexes.

CHAPTER 6

6.0 SUMMARY, CONCLUSION AND RECOMMENDATION

6 1 Summary

Lymphatic filariasis is one of the World's leading cause of permanent and long-term disability. It is a disease that weakens and disfigures a person. About 120 million people in 83 countries of the World are infected with lymphatic filariasis. In the 1950s, 50% of the adult population in The Gambia were infected with lymphatic filarial parasite, which had dropped to 0.0%. Re-infection however cannot be ruled out since the vectors for the disease still remained inhabitants of the country and are widely distributed.

This study looked into anthropophilic mosquitoes in The Gambia for the presence of *Wolbachia* and lymphatic filarial nematode. Pools of anthropophilic mosquitoes were collected from all the divisions in the Gambia using G' rumba method and classified into genera by microscopy. The mosquito pools were pooled into clusters and the DNA extracted using Quick – DNA miniprep plus kit (Zymo) and quantified using a nanodrop spectrophotometer.

Wolbachia DNA was screened targeting the 16srDNA using 16swolbF and 16swolbR specific primers. The supergroups of *Wolbachia* were also determined by targeting the wsp gene using specific primers. *Wolbachia* – mosquito association was determined using bivariate fit of quardratic regression of statistical analysis system (SAS).

The anthropophilic mosquitoes in The Gambia were also screened for the presence of *W.bancrofti* and *B. malayi* using primers specific for the Ssp1 for *W.bancrofti* and Hha1 for *B. malayi.*

Three genera of mosquitoes (*Anopheles*, *Culex* and *Aedes*) were identified with *Anopheles* constituting over 60%. In all the genera of anthropophilic mosquitoes in The Gambia, there is a significant difference in sexual dimorphism with 87.0% being females while male mosquitoes constituted 13.0%.

Wolbachia was detected in the three genera of mosquitoes with an incidence of 34.17% using 16srDNA primers. Only *Wolbachia* supergroup B was identified in the *Culex* and *Aedes* while the *Wolbachia* supergroup could not be determined in *Anopheles* by wsp primers.
W.bancrofti and *B.malayi* were not detected in the DNA of the sampled mosquitoes.

6.2 Conclusion

Anopheles mosquitoes constitutes the predominant anthropophilic mosquito genera in The Gambia. A high proportion of anthropophilic mosquitoes in the country are infected with *Wolbachia*, supergroup B, particularly in *Culex* and *Aedes*. *W.bancrofti* and *B.malayi* DNA were not detected in anthropophilic mosquitoes in The Gambia and hence no active transmission of lymphatic filariasis in the Gambia at the time of this study.

6.3 Recommendation

1. The supergroup of *Wolbachia* in anopheles anthropophilic mosquitoes in The Gambia should be determined by sequencing the 16srDNA amplicons and a blast search.

2. The effect of *Wolbachia* in lymphatic filarial transmission should be further investigated using the strains of *Wolbachia* in The Gambia.

6.4 Contribution To Knowledge

1. A 34.17% incidence of *Wolbachia* infection in anthropophilic mosquitoes in The Gambia has been established.

2. *Wolbachia* in anthropophilic *Culex* and *Aedes* mosquitoes in The Gambia belong to supergroup B.

3. There is no active transmission of filariasis caused by *W.bancrofti* and *B.malayi* during the period of the study.

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APPENDIX I

Number of Anopheles , Culex , Aedes and Mansonia mosquitoes per division

Region	Anopheles	Culex	Aedes	Mansonia
SMD	216	165	30	0
WD	265	84	5	0
NBD	207	81	6	0
LRD	176	68	10	0
CRD	255	129	6	0
URR	180	114	5	0

APPENDIX II

Number of clusters in each division per genara.

For the purpose of cost effectiveness and to increase PCR sensitivity (increase DNA extracted), the sampling points are pooled into clusters (8 sampling points per cluster)

Divisions/ mosquito genera	Anopheles	Culex	Aedes
St Marys (Banjul)	**8**	**8**	**8**
Western Division	**8**	**8**	**2**
North Bank Division	**8**	**8**	**2**
Lower River Division	**8**	**8**	**5**
Central River Division	**8**	**8**	**3**
Upper River Division	**8**	**8**	**4**

Therefore a total 120 clusters for DNA extraction and PCR

APPENDIX III

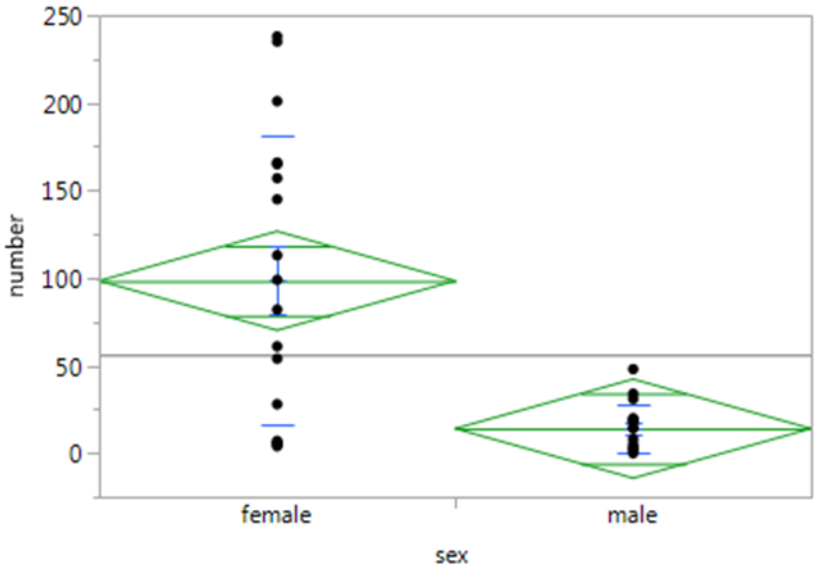
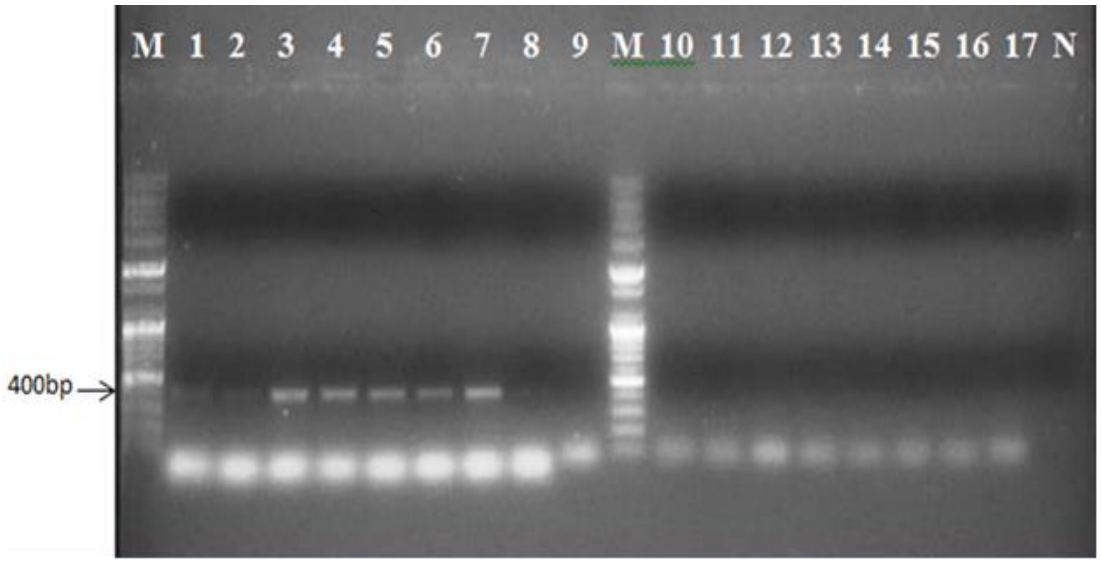


Figure 4.3: Pooled samples of anthropophilic mosquitoes between sexual differentiation in different divisions in the Gambia

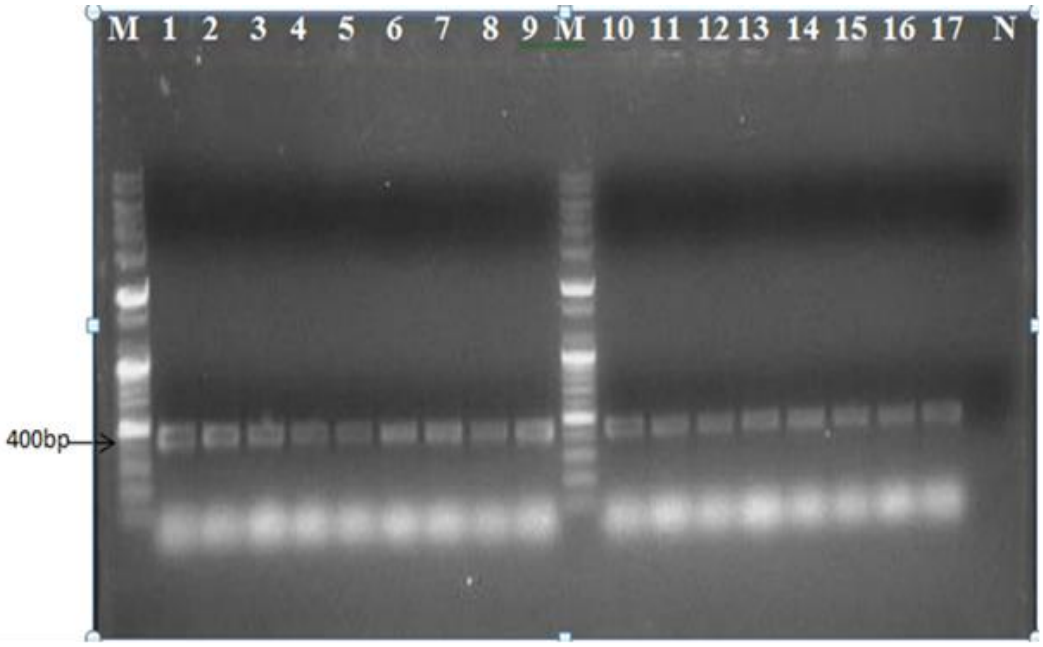
APPENDIX IV



.PCR assay results for wolbachia detection in anthropophilic mosquitoes in the Gambia

M - Molecular weight markers
N - Negative control.
Lanes 1,2,3,4,5,6,and 7 +ve anopheles samples

APPENDIX V



PCR assay results for wolbachia detection in anthropophilic mosquitoes in the Gambia.

M - Molecular weight markers

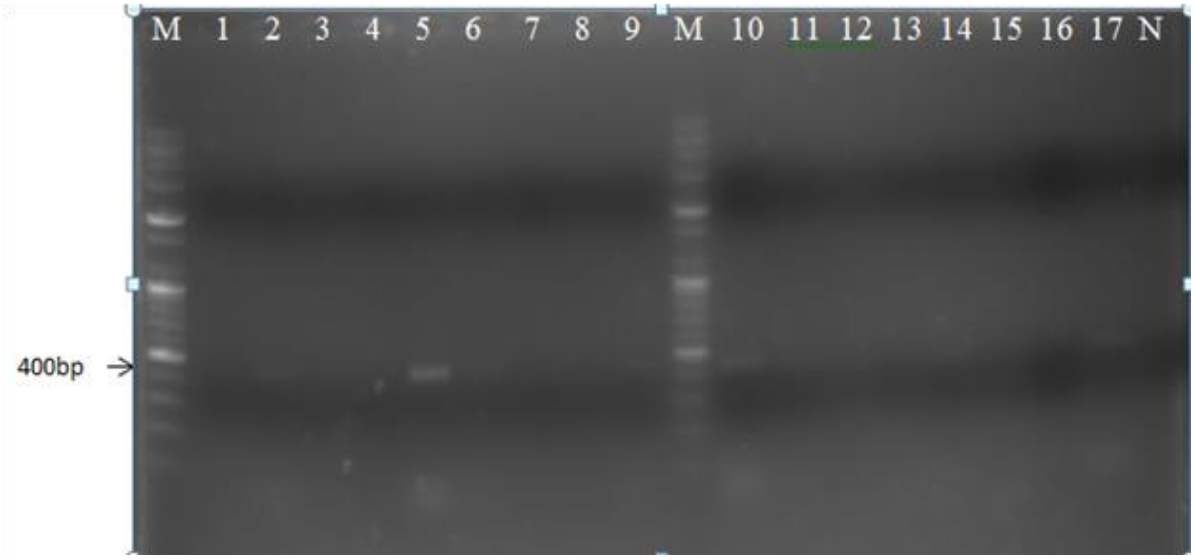
N - Negative control.

Lanes 1,2,3,4,5,6,7 and 8 +ve anopheles samples .

Lanes 9,10,11,12,13,14,15 and 16 +ve in culex samples .

Lane 17 +ve in Aedes samples

APPENDIX VI



PCR results for wolbachia supergroup B

M- Molecular weight marker
N- Negative control
Lanes 2, 5, 6, 9, 10, 12. 14 +ve Wolbachia supergroup B in Culex
Lane 17 +ve Wolbachia supergroup B in Aedes