

**DETECTION AND CHARACTERIZATION OF MOSQUITO-BORNE VIRUSES
CIRCULATING IN MOSQUITOES OF MOROGORO MUNICIPALITY,
TANZANIA**

BELIEVE AHEDOR

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

ABSTRACT

Mosquito-borne viruses primarily infect animals and humans causing significant public and veterinary health threat. Transmitted by hematophagous mosquitoes, dengue virus and Rift Valley fever virus are the most common outbreaks that occurred in parts of Tanzania especially in malaria endemic areas. Several studies reported the presence of the viruses in circulation in animals and humans. Therefore, this study sought to investigate the diversity of mosquito-borne virus vectors and to assess the risk of mosquito-borne viral transmission in Morogoro municipality. Molecular detection of these viruses was carried out on *Aedes aegypti* using reverse transcription polymerase chain reaction (RT-PCR). A total of 7649 mosquitoes comprising of 7224 adults and 424 larvae belonging to five genera (*Aedes*, *Anopheles*, *Culex*, *Eretmapodites*, *Mansonia*) and 14 species were collected. The predominant specie was *Culex quinquefasciatus* 53.9% (n=3891) and *Aedes aegypti* 44.2% (n=3192), most of the species 43.7% (n= 3156) were collected in Mbuyuni, 22% (n=1587) from Kiwanja cha Ndege, 19.2% (n=1387) from Mwembesongo and Mazimbu and Kilakala with 7.6% (n=549 and 545) respectively. About 70% of the *Aedes spp* were collected from used car tyres - the major breeding sites. The mosquitoes' 18S ribosomal ribonucleic acid (rRNA) and viral RNA were successfully amplified, but no specific viruses were detected. However, for the mosquito pools positive for flavivirus, by sequencing the generated PCR products, it was found that there could be false positives due to non-specific amplification of mosquito ribosomal RNA or amplification of arboviral-like sequences integrated into the *Ae. Aegypti* genome. Nonetheless, this result provides an insight into the abundance and distribution of potential vectors in these wards. The close proximity of these vectors to humans poses high risk of virus transmission in the municipality and calls for rational vector control measures.

DECLARATION

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

Believe Ahedor
(Candidate: MSc. One Health Molecular Biology)

Date

The declaration is hereby confirmed by;

Dr. Catherine Walton
(Supervisor)

Date

Prof. Gerald Misinzo
(Supervisor)

Date

COPYRIGHT

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

ACKNOWLEDGEMENTS

I am very grateful to God for giving me life, strength, intelligence and for His grace and mercies throughout the period of my studies.

I thank the Intra-ACP Mobility Scholarship Scheme for funding my studies, and the Noguchi Memorial Institute for Medical Research and the College of Health Sciences, University of Ghana for granting me study leave to pursue my studies.

I extend my sincere gratitude to my supervisors, Professor Gerald Misinzo of Sokoine University of Agriculture and Dr. Catherine Walton of the University of Manchester, UK for their supervision of my research, and most importantly provided me with support under the Africa Award grant funded by the Leverhulme Trust and the Royal Society, and SACIDS-Wellcome Trust to analyse my samples in the UK.

My appreciation also goes to our coordinators; Prof. D. Kambarage and Prof. E. Karimuribo of Sokoine University of Agriculture, as well as Prof. Phyllis Addo of Noguchi Memorial Institute for Medical Research, University of Ghana for their support and guidance. I am thankful to Mr Demetrius A. Max from National Institute for Medical Research, Amani Medical Research Center, Tanga, for the technical assistance during sampling and morphological identification of the mosquitoes and to Ms. Miriam Makange and Fortunate Shija for their assistance in the molecular analysis.

I appreciate my fellow colleagues of the 2014 batch of multinational postgraduate fraternity at the College of Veterinary and Medical Sciences. It has been a great time with you all.

DEDICATION

I dedicate this piece of work to God Almighty for His love and provisions. I also dedicate this work to my parents Mr. Samuel K. Ahedor and Mrs. Victoria K. Ahedor; my siblings Gifty, Godslove, Shepherd and Prosper; and to my good friend Ms. Janet Makafui Akosua Kwame who patiently accepted to have me away for my studies and most importantly for their prayers, guidance, love words of encouragement and support.

TABLE OF CONTENTS

ABSTRACT	ii
DECLARATION	iii
COPYRIGHT	iv
ACKNOWLEDGEMENTS	v
DEDICATION	vi
TABLE OF CONTENTS	vii
LIST OF TABLES	x
LIST OF FIGURES	xi
ABBREVIATIONS AND ACRONYMS	xiii
CHAPTER ONE	1
1.0 INTRODUCTION	1
1.1 Background	1
1.2 Problem Statement and Justification	3
1.3 Research Objectives	4
1.3.1 Overall objective	4
1.3.2 Specific objectives.....	4
CHAPTER TWO	5
2.0 LITERATURE REVIEW	5
2.1 Mosquitoes	5
2.1.1 Mosquitoes as disease vectors.....	5
2.1.2 Taxonomy, habitat and life cycle	5
2.1.3 Medical and veterinary importance of mosquito.....	8
2.1.4 Mosquito identification	9
2.1.5 Mosquito collection methods	10

2.1.6	Mosquito control	12
2.2	Mosquito-borne viruses.....	13
2.2.1	Transmission of MBV	15
2.2.2	The vectors	17
2.2.3	Vector competence.....	18
2.2.4	The classification of MBV	19
2.2.5	Status of MBV in Tanzania.....	25
2.2.6	Diagnostic methods for detection and viral isolation.....	26
2.2.7	Prevention of MBVs.....	28
CHAPTER THREE		30
3.0 MATERIALS AND METHODS		30
3.1	Study Area.....	30
3.2	Sampling Strategies.....	32
3.2.1	Adult mosquito collection	32
3.2.2	Mosquito larvae collection and rearing.....	33
3.2.3	Mosquito preparation and identification	34
3.3	Molecular Detection of Mosquito-borne Virus.....	36
3.3.1	Viral RNA extraction from mosquitoes	36
3.3.2	Complementary DNA (cDNA) Synthesis - (Two – Step).....	36
3.3.3	Viral detection by PCR	37
3.3.4	One-step RT-PCR	40
3.3.5	Agarose gel electrophoresis.....	41
3.4	Sanger Sequencing.....	41
3.5	Statistical Data Analysis	42
CHAPTER FOUR.....		43
4.0 RESULTS		43

4.1 Mosquito Abundance	43
4.1.1 Species richness.....	44
4.1.2 Species diversity.....	47
4.2 Arboviral screening.....	52
CHAPTER FIVE	57
5.0 Discussion	57
CHAPTER SIX	64
6.0 CONCLUSIONS AND RECOMMENDATIONS.....	64
6.1 Conclusions	64
6.2 Recommendations	64
REFERENCES.....	66
APPENDICES	88

LIST OF TABLES

Table 1: Geographical distribution of some MBV identified.....	14
Table 2: Mosquito species sampled from which MBVs were previously isolated	18
Table 3: cDNA Synthesis Mix I for a single sample.....	36
Table 4: cDNA Synthesis Mix II for a single sample	37
Table 8: PCR master mix for arbovirus detection.....	41
Table 9: Number (%) of mosquito species collected and the Simpson's Index of Diversity	45
Table 10: Mosquito distribution (%) by genus between the sampling locations (wards).....	46
Table 11: Number (%) and species of mosquito larvae and pupa collected within Morogoro municipality.	49
Table 12: The habitats of adult mosquitoes and larvae/pupa in Morogoro municipality.....	51
Table 13: The BLAST result of suspected positive flavivirus	56

LIST OF FIGURES

Figure 1: Illustration of the four stages of the life cycle of mosquitoes.....	7
Figure 2: Illustration of transmission and maintenance of mosquito-borne virus.....	16
Figure 3: Infection and transmission process of mosquito-borne virus.	19
Figure 4: Alphavirus genome organization.	21
Figure 5: The Bunyaviridae genome showing the tri-segmented RNA strands.....	23
Figure 6: The flavivirus genome showing the non-segmented RNA strands.	25
Figure 7: Map of Morogoro Municipality showing sampling points and wards.	31
Figure 8: Mosquito traps used in the present study.....	33
Figure 9: Flow Chart of Detection of Mosquito-borne Virus	35
Figure 10: The proportion of mosquitoes by genus sampled from the wards.....	47
Figure 11: Simpson's Index of diversity (A) and specie richness (B) of mosquitoes of Morogoro Municipality.	48
Figure 12: Average number of the different species of mosquito larvae/pupa.....	50
Figure 13: Habitats for adult and larvae mosquitoes.	52
Figure 14: Detection of flavivirus genome in mosquitoes.	53
Figure 15: Detection of dengue virus in mosquitoes.	54
Figure 16: RT-PCR product of the purified suspected DENV.....	54
Figure 17: RT-PCR amplification of mosquitos' 18S rRNA sequence.....	55

APPENDICES

Appendix 1: Extraction of Viral RNA (Modified Qiagen Protocol).....88

Appendix 2: QIAquick Gel Extraction Kit.....90

Appendix 3: GenElute™ PCR Clean-Up Kit - (Sigma-Aldrich).....91

Appendix 4: Mosquito Collection Form92

ABBREVIATIONS AND ACRONYMS

BG	Biogent
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CDC	Centre for Disease Control and Prevention
CHIKV	Chikungunya virus
CO1	cytochrome oxidase subunit 1
DENV	Dengue Virus
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
GPS	global positioning system
IgG	immunoglobulin gamma
IgM	immunoglobulin mega
LD	DNA ladder
MBV	mosquito-borne virus
MoLFD	Ministry of Livestock and Fisheries Development
mRNA	messenger ribonucleic acid
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT-LAMP	reverse transcription loop-mediated isothermal amplification
RT-PCR	reverse transcription polymerase chain reaction

RVFV	Rift Valley fever virus
SIB	Swiss Institute of Bioinformatics
SUA	Sokoine University of Agriculture
TAE	Tris acetate EDTA buffer
URT	United Republic of Tanzania
UV	ultraviolet
WHO	World Health Organization
YFV	yellow fever virus
μl	microlitre

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Mosquitoes are potential vectors of several disease agents that are responsible for both human and animal diseases around the world (Bolling *et al.*, 2015). Depending on the species, female mosquitoes are vectors of protozoa that cause malaria, nematode worms that cause filariasis, and a large number of viruses termed mosquito-borne viruses (MBV). Some of these are dengue virus (DENV), chikungunya virus (CHIKV), yellow fever virus (YFV), West Nile virus (WNV), Rift Valley fever virus (RVFV) and Zika virus (ZIKV) that cause significant public health and socio-economic burden throughout the world, particularly in sub-Saharan Africa (Weaver and Reisen, 2010). The ZIKV has re-emerged as a public health threat in 2015, with its accelerated geographic spread and associated increase in cases of suspected Zika-associated microcephaly in new-borns (Sikka *et al.*, 2016). This has prompted World Health Organization (WHO) to declare a global public health emergency of international concern, and to predict millions of cases likely to occur (WHO, 2016).

Over the years, Tanzania has experienced outbreaks of MBV in most parts of the country. Unpublished records available at the Ministry of Livestock and Fisheries Development (MoLFD) in Tanzania indicate that RVF-like disease in domestic ruminants occurred for the first time in 1930 (Sindato *et al.*, 2014). Several studies have demonstrated the presence of antibodies to RVFV in humans and animals (Mboera *et al.*, 2016). In 2014, WHO reported an outbreak of dengue fever, the worse recorded in both mainland Tanzania and Zanzibar. Prior to this outbreak, Gautret *et al.* (2010) had reported that in Europe, among patients diagnosed with DENV are returning travellers who had a travel

history from Zanzibar. Several other studies have indicated polymerase chain reaction (PCR) detection and serological confirmed antibodies against DENV and CHIKV in children of Kilosa district (Chipwaza *et al.*, 2014), and RVFV among inhabitants of Kilombero district (Sumaye *et al.*, 2015), adjoining districts to the Morogoro municipality.

A number of mosquito species serve as vectors for transmission of mosquito-borne viral disease in nature. Transmission can occur between competent haematophagous mosquito vectors and susceptible vertebrate hosts or through transovarial or venereal in arthropods (DeFoliart and Watts, 1987). The development and survival of these mosquito vectors are closely linked with rainfall events, with very large populations emerging from flooded habitats (Linthicum *et al.*, 1984).

Human infections occur as a result of direct mosquito transmission or, as in the case of RVFV, from aerosol or contact with infected livestock blood or aborted foetal materials (Anyamba *et al.*, 2009). Arboviral infections cause clinical syndromes such as systemic febrile illness, hemorrhagic fever and meningoencephalitis. But most of the viruses cause nonspecific febrile illness with sudden onset of fever accompanied by headache, myalgia, malaise and rash (Gubler, 2001). Infection may lead to a more severe illness, presenting as hemorrhagic fever or meningoencephalitis, which may in some cases prove fatal (Gubler, 2001; Smith *et al.*, 2011).

Due to overlap in clinical presentations, it has often been difficult to distinguish mosquito-borne viral illnesses from other febrile illnesses such as malaria (Gan and Leo, 2013). Unfortunately, despite its severity, there is lack of efficient prophylactic or therapeutic measures except for yellow fever (Bouloy and Weber, 2010). The present study focused on sampling of mosquitoes in the Morogoro Municipality, and using molecular techniques

to screen for the presence of some selected viruses. This would help us to understand the epidemiology of MBV and the associated risk predictors to human exposure. This would further help in the planning and implementing appropriate control measures.

1.2 Problem Statement and Justification

Mosquito-borne viruses are continuously expanding their geographical range into new areas. The spread of these viruses is associated with a number of factors, such as increasing and rapid transportation of people, animals, urbanization and climatic change (which enable certain vector species to conquer regions previously unsuitable), as well as virus genome evolution (Weissenbock *et al.*, 2010). There has been increase in the spread of pathogens and their competent vectors thereby permitting the viruses to break their natural ecology and become established in new geographic locations (Gubler, 2001). Thus, high vector and viraemic populations become increased in these non-endemic areas that could provide conditions for the virus emergence.

Over the past decades, Tanzania has experienced outbreaks of MBV, which continue to occur in some parts of the country. The essential vectors are endemic in the region making the population remain vulnerable and at risk to mosquito-borne viral infections and outbreaks. The impact of such transboundary viral disease on human and livestock populations over the years has been devastating (Gubler, 2009). Their effects include major socio-economic burden such as food security, loss of lives and cost of treatment with the threat of possible outbreaks in any part of the country.

The unexpected spread of the MBV to areas where they have never been recorded necessitated the need to screen mosquitoes for viruses. Early detection will be critical in responding and resolving such outbreaks in the future. This study provides information on

virus activities in the vector population and identified areas that could be at risk were identified in the Morogoro Municipality.

1.3 Research Objectives

1.3.1 Overall objective

To determine mosquito diversity and mosquito-borne viruses in Morogoro Municipality.

1.3.2 Specific objectives

- i To determine mosquito species diversity and ecology in Morogoro Municipal,
- ii To detect MBV and their strains in mosquitoes of Morogoro Municipal,
- iii To identify the specific mosquito species that harbour these viruses.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mosquitoes

2.1.1 Mosquitoes as disease vectors

Mosquitoes are ecologically beneficial for their contribution to biodiversity, food chains and pollination. Some species are a threat to human and animal health because of their role as vectors of disease pathogens (Rueda, 2008). There are about 41 genera and over 3,000 species of mosquitoes (CDC, 2010) and a few of these species are known to transmit diseases. The ability of mosquitoes to carry and support the development of protozoa and viruses probably makes them the most diverse vectors of human disease (Beerntsen and Christensen, 2000).

2.1.2 Taxonomy, habitat and life cycle

2.1.2.1 Taxonomy

Mosquitoes are classified into the order Diptera and family Culicidae. This family includes about 3601 described species and subspecies. The Culicidae family is a large and abundant group that occurs throughout temperate and tropical regions of the world, and even beyond the Arctic circle (Wilkerson *et al.*, 2015). The family is organized into two subfamilies, the Anophelinae (482 species) and the Culicinae with (3119 species). The Aedini group has about 1261 species, and it is the largest of the 11 tribes within Culicinae. The genus *Aedes* in the tribe Aedini is one of three well-known genera of mosquitoes along with *Culex* and *Anopheles* since many of their species are important vectors of pathogens (Wilkerson *et al.*, 2015).

2.1.2.2 Mosquito bionomics and distribution

Mosquitoes have an almost worldwide distribution, being found throughout the tropics and temperate regions. Their survival, density and distribution are influenced by environmental conditions, such as temperature and humidity. Most mosquito vectors require high ambient temperatures for reproduction and this generally occurs in the tropical areas and during summer in subtropical and temperate areas (Gan and Leo, 2013). Although mosquitoes have diverse habitats that allow them to colonize different kinds of environments, they are however absent only from a few islands and Antarctica (Gan and Leo, 2013). They are occasionally carried miles away from their breeding habitat by wind. For instance, *Aedes aegypti*, one of the major vectors of most arboviruses (Gubler, 2001) has been reported to be confined within the tropics and sub-tropics, whereas *Aedes albopictus* occurs in temperate and even cold temperate regions. In recent decades however, *Aedes albopictus* has spread from Asia and has now become established in some parts of Africa, Europe and the Americas (Rueda, 2008).

Mosquitoes can thrive in a variety of habitats such as natural or man-made water bodies except in marine habitats with high-salt concentration. The immature stages can be found in a variety of aquatic habitats, such as ponds, streams, ditches, swamps, marshy areas, temporary and permanent pools, rock holes, tree holes, plant containers (leaves, fruits, husks), artificial containers (tires, tin cans, flower vases), and other habitats (Rueda, 2008).

2.1.2.3 Life cycle

The life cycle of mosquitoes is largely influenced by biotic and abiotic factors. The biotic factors include species, blood meal types, and their natural predation enemies, whereas the abiotic factors include; physicochemical properties of their habitat ie. water type and its

contents, vegetation and the prevailing ecological conditions such as temperature and rainfall (Juliano, 2009). Other complex interactions however, may exist between these factors that may significantly impact their life cycle particularly ecological fitness and vectorial capacity in disease transmission.

The females usually mate only once but produce eggs at intervals throughout their life. Depending on the species, the female adult lays either single eggs as in *Aedes* and *Anopheles* species or in *Culex* in clusters of up to several hundred at a time (Rueda, 2008). Once hatched, it undergoes four distinct stages in their life cycle: egg, larva, pupa and adult (Fig. 1).

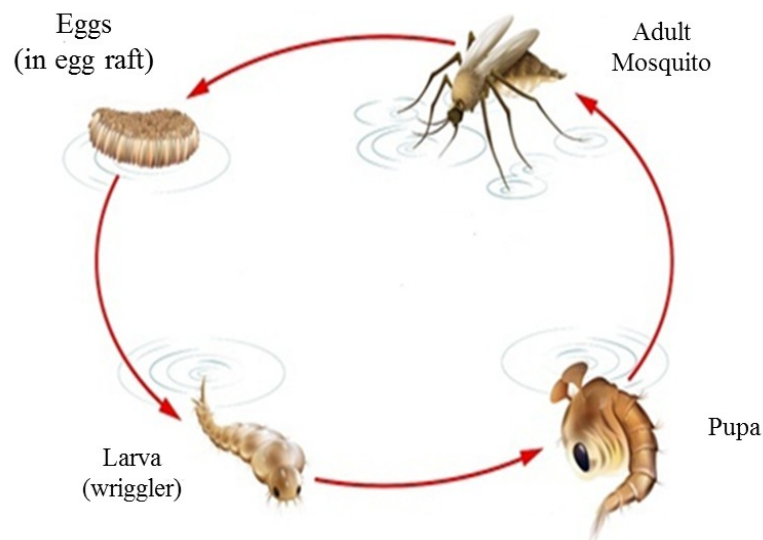


Figure 1: Illustration of the four stages of mosquito life cycle

Source: www.123rf.com/photo - accessed on 26/10/2015

The development of mosquito larvae and pupae require an environment with standing water. In the process of development, the fully-grown larva changes into a comma-shaped pupa. The pupal skin later splits and a fully developed adult mosquito emerge. In warm climates, the larval period lasts about four to seven days or longer (Rueda, 2008). The

pupal period can last between one to three days before becoming adults. Only adult female mosquitoes bite humans and animals (including mammals and birds) in order to obtain a blood meal to produce viable eggs. Male mosquitoes however feed primarily on flower nectars. The entire period from egg to adult takes about 7 -13 days under favourable conditions (Rueda, 2008; Juliano, 2009).

2.1.3 Medical and veterinary importance of mosquito

Mosquitoes have been implicated and found to transmit most medically important diseases that affect man and other animals. They can be said to be the most dangerous insects confronting mankind. They are responsible for the transmission of pathogens and parasites such as protozoans (malaria), nematodes (lymphatic filariasis), and viruses (DENV, YFV, RVFV, CHIKV and ZIKV) which cause serious diseases (Rueda, 2008). Every year, more than three million lives are threatened globally, particularly in the tropics. These have substantially influenced the socio-economic development of communities (Becker *et al.*, 2010).

The haematophagous habit of mosquitoes made them become ideal vehicles for transmitting blood-borne diseases (Ratcliffe, 2004). Transmission involves an obligatory period of replication and development of the pathogen in the vector. Only female mosquitoes bite and suck blood (for successful transmission, multiple blood-meals are necessary). They are able to acquire the pathogens from one vertebrate host and pass them to another. However, the mosquito's ecology and physiology is paramount for disease transmission i.e. highly efficient vectors have to be closely associated with the hosts and their longevity has to be sufficient enough to enable the pathogens to proliferate and for its development into the infective stages in the vector (Becker *et al.*, 2010).

2.1.4 Mosquito identification

2.1.4.1 Morphological identification

Mosquito species vary geographically in their morphological characteristics and biological traits. Identification of the mosquito vectors is one of the important factors in the study of the arboviral diseases. Identification of mosquitoes requires considerable time and experience. Most of the methods employed to identify and classify mosquitoes is largely built around morphological characteristics that distinguishes them from other species (Cook *et al.*, 2005). The light compound microscope and stereomicroscope can be used to distinguish critical characters of larvae and adults, respectively. The observed features are dorsal, ventral, lateral, frontal and caudal of the mosquitoes. Their physiological, behaviour and population biology however are also considered in the identification and classification of new species (Rueda, 2004).

In contrast, the use of morphological identification keys is mainly specific to only a few developmental stages. It is also usually impossible to identify damaged (i.e. when bristles and scales are lost) specimens collected (Kumar *et al.*, 2007). This makes it difficult to identify other stages collected in the field. In addition, morphologically indistinguishable species can be identified by cyto-taxonomic features, using polytene chromosomes, specific to certain tissues in particular developmental stages (Kumar *et al.*, 2007). Owing to the limitations of these techniques, DNA-based technologies for identification can be used.

2.1.4.2 Molecular identification

The DNA-based techniques rely on either DNA probe hybridization or the polymerase chain reaction (PCR). The PCR method enables the use of genetic markers to distinguish between sibling species in defined populations (Becker *et al.*, 2010). The main molecular

genetic marker for species differentiation is the second internal transcribed spacer (ITS2) of the ribosomal gene cluster. However, species identification can be done usually by allele specific amplification (Walton *et al.*, 1999) and, if necessary, by sequencing the amplified products. Application of these methods needs to consider the intraspecific genetic variation, geographic population structure and genetic introgression that may exist between mosquito species (Walton, 1999). This allows precise and efficient identification of the mosquito species.

Another important tool for identification is DNA barcoding. This method has been proposed recently for identifying species in a wide range of animal taxa, using five prime region of mitochondrial cytochrome oxidase subunit one gene (*COI*) gene (Kumar *et al.*, 2007). The *COI* gene serves as the core of a global bioidentification and species biodiversity initiative (Daravath *et al.*, 2013). The unique contribution of DNA barcoding to mosquito taxonomy and systematics is its compressed timeline for the exploration and analysis of biodiversity. According to Knowlton and Weigt (1998), among the mitochondrial genes, *COI* is the most conserved gene in the amino acid sequences and hence has distinct advantage in distinguishing the variation that exist among species.

2.1.5 Mosquito collection methods

Mosquito traps are very effective as a surveillance tool to monitor prevalence and species composition in a specific area. Over the years there have been lots of different kinds of mosquito traps developed, varying greatly in effectiveness and usefulness. The most effective traps use a combination of factors that attract mosquitoes such as light, heat, moisture, carbon dioxide and synthetic chemicals for host attraction, according to the area, the species present and their density (Ndiath *et al.*, 2011). Generally attractants used such as carbon dioxide (CO₂) mimic mammalian scents and body heat providing host cues to

questing female mosquitoes for blood feeding (Corfas *et al.*, 2013). Mosquitoes sampling can be done at different stages of their life cycle; as eggs, larvae, or adults. The larva can be collected by techniques such as netting, dipping, and aspiration. Adult mosquitoes however can be caught by different traps depending on species of interest and as well as their environment.

2.1.5.1 Mosquito trapping devices

The Centre for Disease Control and Prevention (CDC) light trap is a common sampling device used in sampling diverse mosquito species (Amusan *et al.*, 2005). It uses attractant, such as CO₂, that increases catches (Li *et al.*, 2015). The CDC gravid trap however is also designed to collect gravid mosquitoes and other mosquito species during their oviposition (Miller *et al.*, 2015). Other traps such as the Fay-Prince trap come in two forms; the Omni-Directional Fay Prince Trap (dark colour), daytime trap that is quite specific for *Ae. aegypti* and *Ae. albopictus* adults of both sexes (Barrera *et al.*, 2013), and the bidirectional Fay-Prince trap (contrasting shiny black and white colours) that captures more *Ae. aegypti* and *Ae. albopictus* and those of *Culex spp* (Krockel *et al.*, 2006; Barrera *et al.*, 2013). Others are the Biogents-Sentinel traps (BGS) which has BG-Lure, a combination of lactic acid, ammonia, and caproic acid bait that create an air current that mimic that of the human body (Englbrecht *et al.*, 2015). Shannon trap on the other hand is also used to capture adult mosquitoes (Cavallari *et al.*, 2014). These traps are employed for catch diversity of mosquito species.

2.1.5.1.1 Hoover traps

Hoover trap is a hand-held battery-operated (12-volt) device with a long pole. The battery is held in a backpack and connected to the device. The device has a rotor/fan that generates a reverse (negative) pressure in the chamber and can suck in mosquito species

and any other insects into the capture cup (Fig. 8). This trap is useful for surveillance studies because it is versatile and catches the mosquitoes alive, unharmed and potentially also for blood fed mosquitoes for host identification. The main disadvantage just like the CDC light traps is, it attracts other non-target species, which are drawn into the capture net (Amusan *et al.*, 2005).

2.1.6 Mosquito control

One most common strategy in mosquito control has always focused exclusively on the use of synthetic insecticides. Insecticides such as larvicides are used for permanent habitats (ponds, lakes), and adulticides for indoor residual spraying (Raval-Nelson *et al.*, 2005). However, the uses of these public health and agricultural insecticides have had a negative impact on non-target organisms and the environment. This has led to a substantial increase in physiological mosquito resistance, toxicity problems to humans, environmental contamination, ecological imbalances and economic burden (Rueda, 2008). The World Health Organization encourages alternative integrated vector control approach that is environmentally friendly (WHO, 2004).

There is much interest in insect pathogenic fungi because they are considered to offer an environmentally friendly alternative to chemical pesticides. These hyphomycetous insect-pathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana*, are produced commercially and are used against several agricultural insect pests worldwide (Scholte *et al.*, 2005; Lwetoijera *et al.*, 2010; Kamareddine, 2012; Xiao *et al.*, 2012). They produce a variety of hydrolytic enzymes e.g., proteases, lipases, and other factors that promote germination and growth of the fungus on the surface of the mosquitoes. As it penetrates into its cuticular layers, the host integument and encounters the host immune system dies (Xiao *et al.*, 2012).

Naturally occurring bacteria are also used to control mosquito larvae. The application of this microbial insecticide; delta-endotoxins is derived from *Bacillus thuringiensis israelensis* and *Bacillus sphaericus*. These bacteria produce endotoxin crystals during sporulation which when ingested by the larvae hydrolyzes its epithelial cells and the larvae die within 24 hours. It is highly specific and is safe to most of the non-targeted aquatic vertebrates and invertebrates. However *Bacillus thuringiensis israelensis* is effective on *Anopheles* larvae, and *B. sphaericus* is more effective against *Culex* larvae (Ratcliffe, 2004; Kamareddine, 2012).

Other genetic strategies have been developed using classical genetics, such as the sterile insect technique (SIT). This method relies on the release of large numbers of sterile males that mate with wild females by so doing decrease the females' reproductive potential, hence the target population will decline and collapse (Alphey *et al.*, 2013). Several approaches have been described for making mosquitoes refractory to malaria including either the expression of specific antibodies (Isaacs *et al.*, 2012), peptides (Ito *et al.*, 2002) or cell signalling manipulation (Corby-Harris *et al.*, 2002). However, in *Aedes* mosquitoes that transmit arboviruses, RNA interference (RNAi) has been used as a mechanism for suppressing virus replication (Alphey *et al.*, 2013). A maternally inherited intracellular bacterium *Wolbachia pipientis* is used to drive refractory genes into vectors (Hoffmann *et al.*, 2011; Alphey *et al.*, 2013) that can disrupt reproduction with noninfected sperms. Ultimately, if a refractory male mosquito is infected with any particular virus be released to replace the naturally susceptible ones, the susceptible genes of the diseases in the mosquito population become neutralised (Alphey *et al.*, 2010).

2.2 Mosquito-borne Viruses

Mosquito-borne viruses have become of high public and veterinary health significance. It has been estimated that, more than 550 MBVs have been identified, of which more than

130 species are known to cause infectious diseases in humans and animals (Gao *et al.*, 2010). They are transmitted primarily by mosquitoes but ticks can also transmit arboviruses (Gubler, 2009). With the exception of the deoxyribonucleic acid (DNA) containing tick-borne African swine fever virus (ASFV) of the family *Asfarviridae*, all known arboviruses are ribonucleic acid (RNA) viruses (Labuda and Nuttall, 2004).

A significant subset of these viruses, include members of the *Flaviviridae*, *Bunyaviridae*, and *Togaviridae* families. The most important MBVs include flaviviruses (*Flaviviridae*) - DENV, YFV, West Nile virus (WNV); alphaviruses (*Togaviridae*) - CHIKV, o'nyongnyong virus (ONNV), Sindbis virus (SINV); and bunyaviruses (*Bunyaviridae*) such as RVFV (Labeaud, 2008; Labeaud *et al.*, 2011; Dash *et al.*, 2013; Kean *et al.*, 2015). They are transmitted widely in different parts of the world. The most important reservoir hosts for MBV are birds, rodents, primates and humans (Table 1).

Table 1: Geographical distribution of some MBV identified

Pathogens	Disease	Animal Host/ Reservoir	Geographic distribution
<i>Togaviridae</i>	Chikungunya	Primate,	Africa, Asia
	Onyongnyong	humans	Africa
	Sindbis fever	Not Known Birds	Asia, Africa, Australia, Europe, Americas
<i>Flaviviridae</i>	Dengue fever	Primates,	Worldwide in tropics
	(serotypes 1-4)	humans	Africa, South America
	Yellow fever	Primates,	Africa, Asia, North
	West Nile encephalitis	humans Birds	America, Europe
<i>Bunyaviridae</i>	Rift Valley fever	Not known	Africa
	Crimean-Congo	Rodents, sheep	Europe, Asia, Africa
	haemorrhagic fever		

Source: Gubler (2009)

2.2.1 Transmission of MBV

Mosquitoes are by far the most important primary vectors of arboviruses. Aves and vertebrates are the most important vertebrate reservoir hosts (Gubler, 2001). Most MBVs are principally maintained in nature and cycle horizontally with transmission during blood feeding by competent haematophagous mosquitoes, while a few are maintained by vertical transmission (transovarian) from adult mosquito to offspring or through venereal transmission during copulation (DeFoliart and Watts, 1987; Coffey *et al.*, 2013). However, transmission of the virus largely depends on some factors such as susceptible vertebrate hosts, the vector life cycles, vector competence and being in a suitable environment where there is enough biting activity by the vectors (Gubler, 2001; Afrane *et al.*, 2005).

2.2.1.1 Mosquito-borne virus transmission cycle

In arboviral transmission, competent vectors and vertebrate hosts must intersect repeatedly within a permissive environment. Viral concentration must however, exceed a threshold for infection and development of viremia to be established (Weaver and Reisen, 2010). It is however reported that most mammalian hosts develop immune responses that prevent reinfection (Kuno and Chang, 2005). Thus, vertebrates' immunity and a reduced susceptible population size are determinants for transmission.

In enzootic transmission, the viruses are maintained in complex cycles involving non-human primates and other vertebrates and mosquito vectors eg *Aedes* mosquito in sub-sylvatic foci cycle (Fig. 2). These cycles are usually undetected in nature, pointing to the fact that, epidemics/epizootics in humans and domestic animals usually occur only after spillovers from the primary cycle into the peridomestic environment by a bridge vector (Gubler, 2001). Transovarial or vertical transmission (Fig. 2) can also occur. This serves as a sustainable survival mechanism for the virus for generations (Romoser *et al.*, 2011; Coffey *et al.*, 2013).

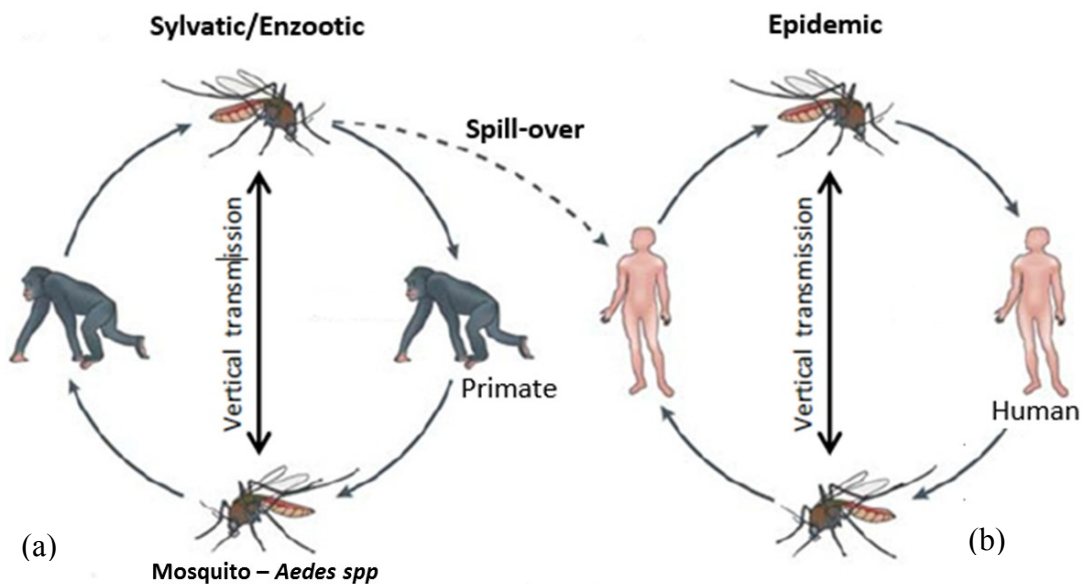


Figure 2: Illustration of transmission and maintenance of MBV.

Source: Whitehead *et al.* (2007)

Although a bite from an infected mosquito may cause an infection, several factors such as vector competence, susceptibility and viraemic maintenance of the host, and ecological interactions between hosts and vectors will influence viral transmission. This multistage transmission cycle constrains viral evolution and may limit viral fitness (Coffey *et al.*, 2013).

According to Gubler (2001), many arboviruses may have more than one vertebrate host or arthropod vector. But a few of these viruses, such as DENV, YFV, and CHIKV, cause high viremia levels in humans and may be transmitted from person to person by a vector, usually a mosquito, which often, but not always, signals epidemic transmission. However, Weaver and Reisen, 2010 reported that the introduction of new strains can increase virulence and viremia levels in vertebrates, thereby expanding host range and increasing amplification potential.

2.2.2 The vectors

Vectors of MBVs constitute a potent and constant threat to humans and livestock, and not all mosquito species can transmit the virus. Over 300 species of mosquitoes notably *Aedes spp.*, *Mansonia spp.*, *Anopheles spp.* and *Culex spp.* are implicated to transmit viruses (Lutomiah *et al.*, 2013; Liang *et al.*, 2015). The *aedine* mosquito species particularly *Aedes aegypti* and *Aedes albopictus* have been reported to harbour viruses like DENV, CHIKV, YFV and RVFV. Others are *Anopheles spp* such as *Anopheles funestus*, *An. gambiae* reported to be vectors of ONNV and CHIKV. The BUNV and CHIKV have also been isolated from *Culex pipiens* and other *Culex spp*, and also from *Mansonia africana* and *Mansonia uniformis* but can also transmit RVFV (Mutebi *et al.*, 2012; Kean *et al.*, 2015) as shown in Table 2.

Table 2: Mosquito species sampled from which MBVs were previously isolated

Mosquito Species	Mosquito-borne Virus (MBV)
<i>Aedes aegypti</i>	CHIKV, (DENV 1-5), WNV, YFV, ZIKV
<i>Aedes simpsoni</i>	YFV
<i>Anopheles coustani</i>	CHIKV, WNV
<i>Anopheles gambiae</i>	CHIKV, ONNV, ZIKAV
<i>Culex antennatus</i>	WNV, RVFV
<i>Culex cinereus</i>	CHIKV
<i>Culex decens</i>	WNV, CHIKV
<i>Culex quinquefasciatus</i>	CHIKV, WNV
<i>Culex tigripes</i>	SINV
<i>Culex zombaensis</i>	BUNV, RVFV
<i>Eretmapodites quinquevittatus</i>	RVFV
<i>Mansonia africanus</i>	BUNV, CHIKV, RVFV, WNV

Chikungunya virus (CHIKV); Onyongnyong virus (ONNV); Rift Valley fever virus (RVFV); West Nile virus (WNV); Sindbis Virus (SINV); Yellow Fever virus (YFV); Zika virus (ZIKV) reported by Mutebi *et al.* (2012) and Dengue virus (DENV 1-5) by Mustafa *et al.* (2014).

2.2.3 Vector competence

Vector-borne infections require a vector to be able to acquire a pathogen, maintain, and successfully transmit it to a susceptible host. It is a complex process influenced by the following factors; the external factors which include temperature, availability of vertebrate hosts and vector population density, whereas internal factors are mosquito survival and virus replication abilities (Coffey *et al.*, 2014). The viral pathogen must overcome several obstacles before being transmitted to another host. This depends on intrinsic factors which include the internal physiology and innate behavioral traits of the vector, and its ability to transmit an agent as well as feeding duration and host preferences (Agarwal *et al.*, 2014).

During the extrinsic incubation period (Fig. 3), Coffey *et al.* (2014) explained that the virus from viraemic vertebrate hosts during blood feeding must be able to evade the gut wall, survive in arthropods' tissues, such as hemolymph, muscles, or the reproductive system. It must be able to penetrate the salivary glands for injection into a new host.

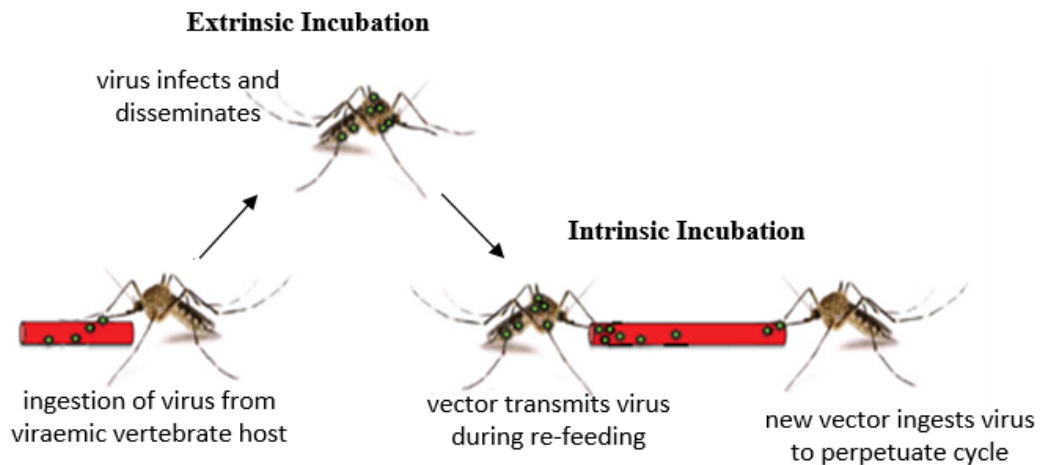


Figure 3: Infection and transmission process of mosquito-borne virus.

Source: Coffey *et al.* (2014)

Transmission of the virus to vertebrate hosts is achieved by expectorating the virus in saliva. New re-feeding vectors thus perpetuate the cycle by ingesting virus during intrinsic incubation, a period of viremia in the vertebrate host (Coffey *et al.*, 2014). Some of the extrinsic factors for efficient vector-borne disease transmission include longevity of the mosquito, host feeding preferences, abundance of mosquitoes, climatic conditions, and genetic variation in the infectivity of the pathogen. The horizontal transmission through saliva that is injected when a mosquito feeds on blood is the common mechanism of transmission, and vertical transmission via infected eggs may also occur (Agarwal *et al.*, 2014; Coffey *et al.*, 2014).

2.2.4 The classification of MBV

2.2.4.1 The family *Togaviridae*, genus *Alphavirus*

Alphaviruses remain important emerging mosquito-borne, zoonotic pathogens that causes both localized human outbreaks and epizootics. It is one of two genera in the family

Togaviridae. It is classified on the basis of antigenic properties and has nine viruses of medical and economic importance (Schmaljohn and McClain, 1996). These viruses are globally dispersed and include Venezuelan Equine Encephalitis virus (VEEV), Eastern Equine Encephalitis virus (EEEV), and Western Equine Encephalitis virus (WEEV) prevalent in the America. The rest are Ross River virus (RRV) found in Australia, Chikungunya virus (CHIKV) prevalent in both Asia and Africa, and O'nyong-nyong virus (ONNV) in Africa (Atkins, 2013).

Transmitted by blood-sucking arthropods, the virus replicate in both arthropod and vertebrate hosts (Powers and Roehrig 2011). PIALOUX *et al.* (2007) reported that CHIKV, one of the most important *Alphaviruses* of human pathogenicity was first isolated in Tanzania in 1953 during an outbreak of human febrile illness accompanied by severe arthralgia and rash. There are however three genotypes of CHIKV known, and include the West African, Asian and the 'East, Central and South African' genotypes (Strauss and Strauss, 1994; Gorchakov *et al.*, 2008).

2.2.4.1.1 The alphavirus genome

Alphavirus are enveloped single-stranded positive-sense RNA genome. Its virions are spherical, 60 to 70 nm in diameter (Schmaljohn and McClain, 1996). Its genome is about ~11.7 kb long and contains two regions, a non-structural domain making up the five prime two-thirds of the RNA (Fig. 4), and a structural domain at the three prime end making up the remaining one-third of the genome (Strauss and Strauss, 1994).

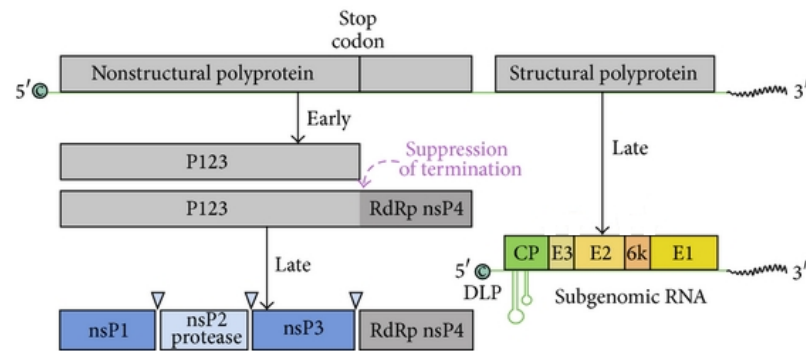


Figure 4: Alphavirus genome organization.

Source: Hernandez *et al*, 2014

The non-structural domain encodes four viral non-structural proteins (nsP1- nsP4), which are essential for replication and polyprotein processing (Fig. 4). In addition to copying the RNA genome, the non-structural proteins synthesize 26S sub-genomic mRNA which is capped and polyadenylated, ultimately produces five individual structural proteins which consist of the viral capsid (C), two envelope glycoproteins (E1 and E2), and two peptides (E3 and 6K) (Strauss and Strauss, 1994).

2.2.4.2 The Family *Bunyaviridae*, Genus *Phlebovirus*

The *Bunyaviridae* family comprised of five genera based on morphological, serological and biochemical characteristics. These include *Hantavirus*, *Nairovirus*, *Orthobunyavirus*, *Tospovirus*, and *Phlebovirus* (Horne and Vanlandingham, 2014). Three of those genera namely *Orthobunyavirus*, *Nairovirus*, and *Phlebovirus* are transmitted by hematophagous arthropods (e.g. mosquitoes, ticks, sand flies); *hantaviruses* are transmitted among rodents (Horne and Vanlandingham, 2014). Their associated arthropods not only serve as vectors, but also as virus reservoirs in many cases. Several viruses of this family can produce mild to severe disease in humans and animals (CDC, 2013).

Rift Valley fever virus (RVFV) belongs to the genus *Phlebovirus* and it is a viral zoonotic disease that affect ruminants and humans. The incubation period ranges from one to three days followed by fever, recumbency, and haemorrhagic diarrhea (CDC, 2013). High rates of abortion are also associated with epidemics of Rift Valley haemorrhagic fever (RVHF), often described as abortion storms. Johnson *et al.* (2012), reports that mortality can reach 70%. The virus however, causes disease in humans ranging from influenza-like illness to haemorrhagic fever with hepatomegaly and encephalitis (Bouloy and Weber, 2010).

Isolated first in the Rift Valley in Kenya in 1931, it is currently endemic in Kenya, and Tanzania (Nderitu *et al.*, 2011), South Africa and the Middle East (Bird *et al.*, 2007). There has been a reported outbreak of RVFV in the Arabian Peninsula associated with livestock movements from East Africa (Fagbo, 2002). This virus has been confirmed in both humans and livestock as its epidemics continue to seriously have economic implications on veterinary and human health, although clinical disease occurs.

It is transmitted principally by *Aedes spp.* and it's believed to maintain the virus but other species such as *Culex*, *Anopheles*, *Eretmapodites*, and *Mansonia spp.* are also implicated to transmit (Mutebi *et al.*, 2012). It has also been isolated from midges, black flies, and ticks as well (Pepin *et al.*, 2010). RVFV was also isolated from the adult mosquitoes reared from collected larvae and pupae a possible transovarial transmission (Himeidan *et al.*, 2014). However, humans can also be affected through contact with blood or aerosol from infected animal tissues.

2.2.4.2.1 The *Bunyaviridae* Genome

Bunyavirus is a tri-segmented negative-stranded RNA linear genome; L (large) segment range between 6.8 kb and 12 kb, M (medium) segment between 3.2 kb and 4.9 kb, and S (small) segment between 1 kb and 3 kb (Fig. 5). All members of the family encode four structural proteins i.e. two glycoproteins called Gn and Gc according to their position within the primary gene product, a nucleoprotein, N that encapsidates the genomic RNA segments and an RNA-dependent RNA polymerase, called L protein (Blakqori *et al.*, 2012).

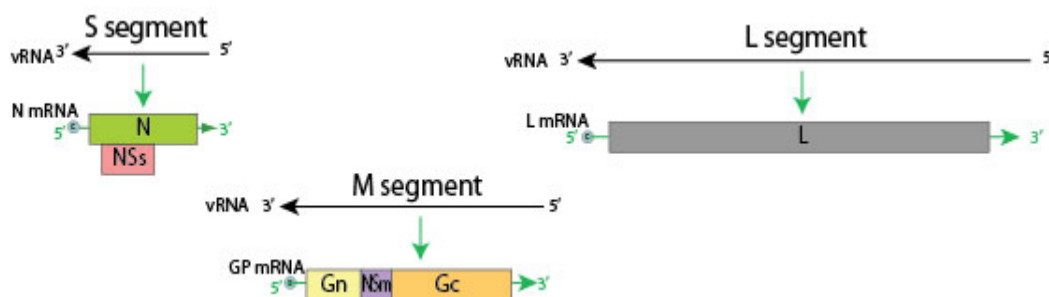


Figure 5: The *Bunyaviridae* genome showing the tri-segmented RNA strands.

Source: (SIB, 2015)

2.2.4.3 The family *Flaviviridae*; Genus *Flavivirus*

The genus *flavivirus* is a member of the family *flaviviridae* comprising about 39 defined members belonging to the MBVs. The globally most important pathogens of this genus include, YFV, DENV, JEV and WNV. Zika virus (ZIKV) also belongs to the flavivirus which causes haemorrhagic fever and encephalitis in humans declared by W.H.O as a global health threat (WHO, 2016). Ticks or mosquitoes either transmit these viruses but vertebrate serve as reservoirs. The virus has a forest cycle with lower primates as hosts and *Aedes* mosquitoes as their principal vectors (Weaver and Reisen, 2010). Nonetheless, only some particularly *Aedes aegypti* vectors have fully adapted to human-urban

environment and no longer require the forest cycle for maintenance (Gubler, 2002; Weaver and Reisen, 2010). As a result, inter-human transmission is highly effective due to the presence of domestic vectors and relatively high viremia titres in infected persons and their susceptibility (Harrington *et al.*, 2001).

Flavivirus caused by mosquito are found on all continents except Antarctica (Weissenböck *et al.*, 2010) and unlike other flavivirus, DENV are not zoonotic, but exclusively utilize humans as reservoir and amplification host. There are now five antigenically distinct serotypes (DENV 1 - 5), and although they share identical epidemiological features, they are genetically distinct (Mustafa *et al.*, 2014). The impact of DENV infections on human health is enormous. It is the most important human MBV pathogen, with an estimated 50 - 100 million cases annually (Weaver and Reisen, 2010). These symptomatic infections commonly manifest as a self-limiting flu that is characterized by a sudden onset of fever, arthralgia, myalgia, retro-orbital headaches and leucopenia. Infections show little or no subclinical signs and symptoms with a fatality rate of about 5% (Kyle and Harris, 2008).

2.2.4.3.1 The *Flavivirus* Genome

All members of the *flaviviridae* family are small, enveloped viruses that contain a non-segmented, positive-sense, single stranded RNA genome, approximately 11 kb in length (Lindenbach and Rice, 2003). Mature viruses have a diameter of about 50 nm and a single open reading frame encodes three structural protein, the capsid (C), membrane (M), and envelope (E), and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (Lindenbach and Rice, 2003).

The polyprotein is translationally cleaved by host proteases and the viral serine protease NS2B/NS3 (Fig. 6). The capsid protein is associated with the viral RNA forming the

nucleocapsid, while the viral envelope contains the prM/M and the E proteins. The E protein serves as the major flavivirus antigenic determinant and is involved in the attachment and entry of the virion into the cell. The prM protein is essential for proper folding of the E protein and is cleaved to M by furin prior to release of the mature virion from the cell (Lindenbach and Rice, 2003).

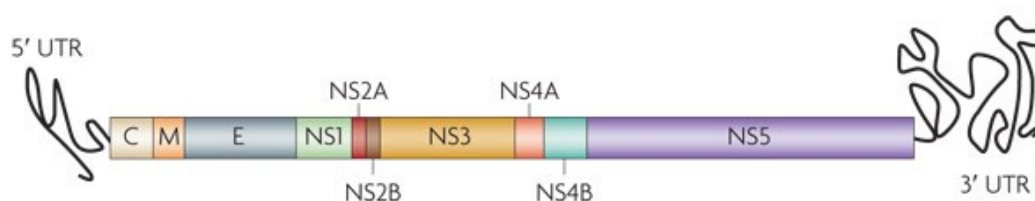


Figure 6: The *flavivirus* genome showing the non-segmented RNA strands.

Source: Guzman *et al.* (2010).

The genomic RNA is capped and serves as mRNA for all proteins. Structural proteins are encoded at the five prime end of the genome, and non-structural proteins are encoded in the three prime.

2.2.5 Status of MBV in Tanzania

Tanzania has experienced several mosquito-borne virus epidemics in different parts of the country. Dengue virus was reported in 2010 among returning travellers in Europe, particularly patients with a travel history to Zanzibar, Tanzania (Gautret *et al.*, 2010). Four years later, in May 2014, Tanzania experienced the worst dengue fever outbreak that has claimed lives. This outbreak was confirmed in seven regions on the mainland, and two other regions in Zanzibar (WHO, 2014). Mosquitoes collected during the outbreak particularly *Aedes aegypti* have been reported to harbour DENV (Mboera *et al.*, 2016).

Rift Valley Fever virus (RVFV), was first reported in 1930. Subsequently, several studies demonstrated the presence of antibodies to RVFV in humans and animals in northern Tanzania including Manyara, Tanga, Dodoma, Morogoro, Dar es Salaam, Iringa, Mwanza

and Singida regions (Nderitu *et al.*, 2011), also among inhabitants of Kilombero (Sumaye *et al.*, 2015). Kigoma district however recorded RVFV for the first time in four years after the 2007 epizootic in Tanzania in livestock (Kifaro *et al.*, 2014). Dengue virus and CHIKV have also been reported in children in Kilosa (Chipwaza *et al.*, 2014) in Morogoro region. Crump *et al.*, (2013) also reported an acute CHIKV infections in a clinical study in Northern Tanzania confirmed by PCR. These studies and many others points to the presence of the virus affecting both human and livestock population suggesting that these viruses have become established.

2.2.6 Diagnostic methods for detection and viral isolation

In the absence of a vaccine or any specific drug for the treatment of mosquito-borne viral infections, an early diagnosis is essential. In the detection and isolation of mosquito-borne viral infections, cell cultures, serological and molecular methods are utilized. Virus isolation in cell cultures has long served as the “gold standard” for virus detection, and it is the method to which all others have been compared (Leland and Ginocchio, 2007). However, in recent years, technological advances, ranging from the development of monoclonal antibodies to the introduction of molecular diagnostics, have provided powerful tools to use in attempting to detect the presence of viral infections. Molecular detection of viral DNAs and RNAs and molecular amplification by RT-PCR and other techniques such as real-time loop-mediated isothermal amplification (RT- LAMP) are sensitive and highly specific in viral identification.

2.2.6.1 Cell culture method

Virus isolation is carried out in cell lines, preferable mosquito cell lines. Its choice however depends on the availability of a host-cell cultures that serve as an indicator of virus infection i.e., its cytopathic effects (Samuel and Tyagi, 2006). The cell lines also support the multiplication of very low virus titre that can be detected using indirect immune-

fluorescent technique (IFA). This technique is an important tool in the detection of virus antigen with its simplicity and specificity (Gubler and Trent, 1994; Thenmozhi *et al.*, 2014). In their work, Ahmad *et al.*, 1997 detected DENV using cell culture (C6/36 clone) of *Ae. albopictus* by peroxidase staining in which the positive isolates were further confirmed by the RT-PCR.

2.2.6.2 Serological method

The serological method employs enzyme-linked immunosorbent assay (ELISA) in detection of virus-specific antibodies of a particular virus. This method is the most widely used in routine practice and it is shown to be a sensitive alternative to insect bioassay for monitoring arboviruses in wild mosquito population (Samuel and Tyagi, 2006). This technique was used in dengue virus surveillance for monitoring the dengue viral activity in endemic areas (Thenmozhi *et al.*, 2014). Despite its numerous advantages, it however lacks specificity that is due to cross-reactivity between related viruses (Hall *et al.*, 2012).

2.2.6.3 Molecular methods

Molecular techniques for detecting nucleic acids have revolutionized the diagnosis of several viral diseases. A range of highly sensitive nucleic acid based molecular tests have been developed for the diagnosis of MBV infections in experimentally and naturally infected mosquitoes. This includes RT-PCR, nested RT-PCR methods, quantitative real-time PCR (qRT-PCR), multiplex PCR-based microarray assay and RT-Loop-mediated isothermal amplification (RT-LAMP) (Mansfield *et al.*, 2015; Drosten *et al.*, 2002). By use of next-generation sequencing technologies, unidentified viral isolates and genetic characterization of new vector-borne viruses are rapidly analysed (Hall *et al.*, 2012).

Real-time PCR can be used to detect specific viral nucleic acid in vector populations (Hall *et al.*, 2012). This methods has been applied using universal primers and enzyme polymerase in a thermal cyclers to target the viral gene and to detect a range of viruses (Johnson *et al.*, 2010). On the other hand, a one-step reverse transcription RT-LAMP assay can be used to amplify nucleic acid with high specificity, efficiency, and rapidity under isothermal conditions with a set of primers to detect virus. The procedure is very simple and rapid and does not require thermal cycling instrumentation (Mansfield *et al.*, 2015). Detection of the amplified nucleic acid is done by real-time fluorescent-labelled molecular beacon probes or by visual inspection when electrophoresed on an agarose gel.

The detection threshold of the RT-LAMP assay was found to be similar and more sensitive to RT- PCR method (Parida *et al.*, 2004; Soliman and El-Matbouli, 2006). This has been adapted to dengue virus detection with sensitivity near that of virus isolation in cell cultures (Shu and Huang, 2004) and also to detect and quantify CHIKV in the acute phase of infection on the field (Lakshmi *et al.*, 2008).

2.2.7 Prevention of MBVs

There are no specific anti-viral treatment or efficient prophylactic available except for yellow fever vaccine (Bouloy and Weber, 2010) although there are vaccine candidates for DENV and RVFV under clinical trial evaluation (CDC, 2013). A preventive measure among other steps is the use of personal protective wears to avoid mosquito bites. Thier (2001), points out that measures such as the use of biological controls and biopesticides are aimed at reducing the vector populations. One other measure is to get rid of water holding containers such as buckets, tins, car tyres that serve as a conducive common breeding sites (Mboera *et al.*, 2016). However, continuous surveillance is necessary as well as public education.

Studies have shown that virus infection and transmission in mosquitoes can be impeded by co-infection with *Wolbachia pipientis*. This is a strain of bacterium that can trans-infect *Aedes* mosquitoes and inhibit the replication and dissemination of RNA viruses, including DENV (Hoffmann *et al.*, 2011). This discovery suggested a potential strategy to interfere with transmission of arboviruses by artificially infecting mosquitoes with *Wolbachia* (Hoffmann *et al.*, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The present study was conducted in Morogoro Municipality in Morogoro region, Tanzania. All sampling points within the study area were recorded using global positioning system (GPS) and mapped (Fig. 7). The municipality lies between latitude 06.4915 south of the equator and longitude 037.3940 to the east, with an elevation of 504 meters above sea level. It occupies a total of 260 square kilometres land coverage with a population of about 315 866 (URT, 2013).

The climate of the region can be described as tropical to sub-humid with four main seasons: hot-wet season from December to March, cool-wet season from April to June, cool-dry season from July to August and hot-dry season from September to November. The cool-dry season records a temperature as low as 12 °C averagely while a temperature of 38 °C is observed during the hot-dry seasons. Annual rainfall in the region ranges from 1 200 – 1 400 mm (URT, 2013).

The sites were selected based on some ecological and demographical characteristics such as adult and larval habitats, the topography, vegetation and areas of high human habitation. The municipality has moderately dense human population with hotels, tertiary institutions and industries, which attracts many people from all walks of life. Sampling was conducted in Mazimbu, Kiwanja cha Ndege, Mwembesongo, Mbuyuni, and Kilakala wards (Fig. 7). Kiwanja cha Ndege is a business area characterized by high standard dwelling with a number of garages. So is Mwembesongo which has vegetation cover with high-populated neighbourhoods. Located in this ward is the busiest bus terminal.

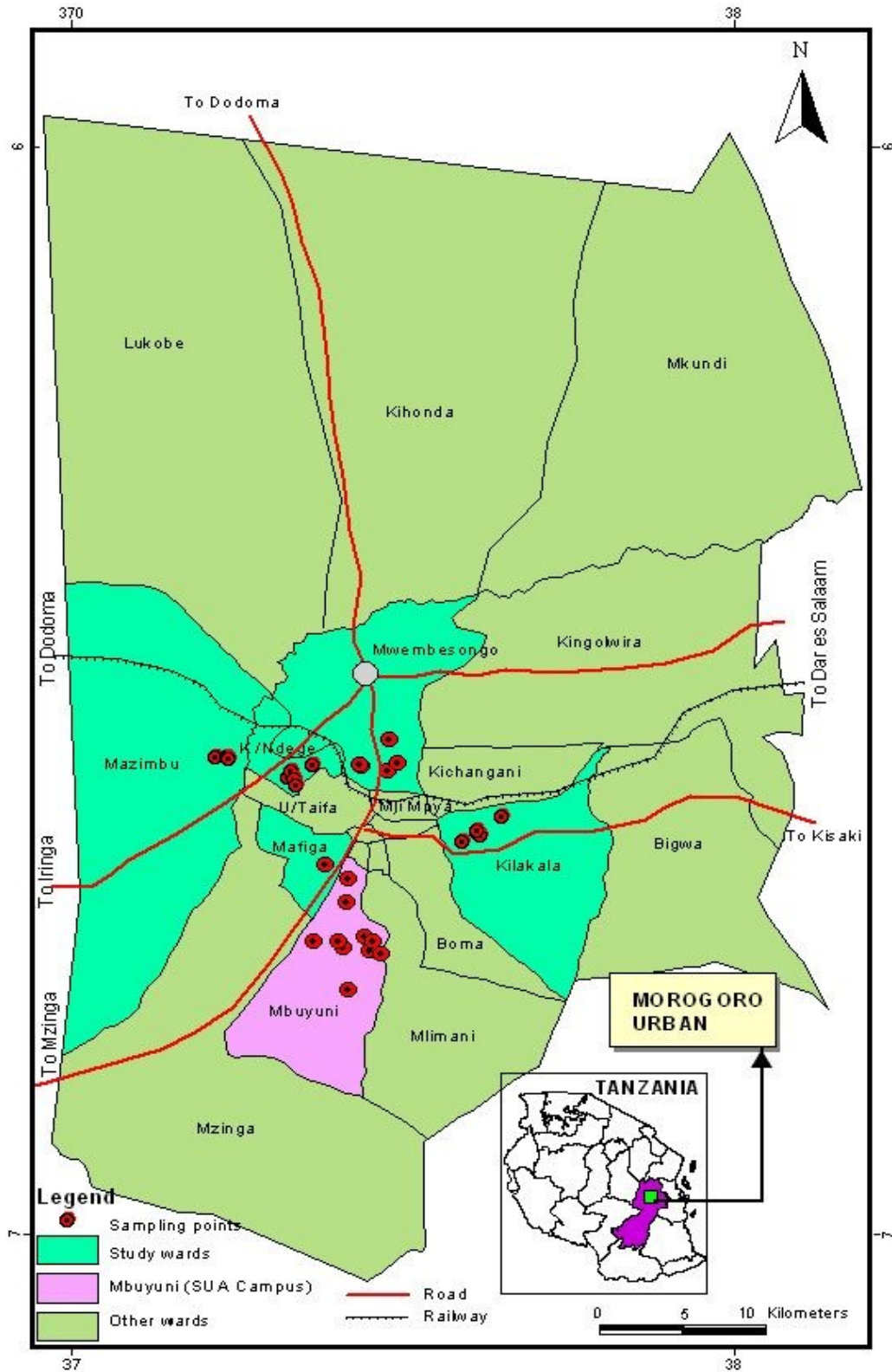


Figure 7: Map of Morogoro Municipality showing sampling points and wards.

Source: Arc GIS 10.3 Version 2015

Kilakala is a middle-class residential area, with high-density population. Some government institutions such as regional hospital and prisons service are located in this ward. The area is characterized by vegetation. Mbuyuni is where the Sokoine University of Agriculture is located and are surrounded by rural communities at the peripheral of the Uluguru Mountain characterized with low human population but with high vegetation.

3.2 Sampling Strategies

A cross-sectional entomological investigation was carried out to sample both adult mosquitoes and their larvae. Sampling was done successfully from five wards in the Morogoro Municipality in Morogoro region. Mosquito collection was carried out between December 2015 and January 2016. Mosquito sampling forms were used (Appendix IV) to record ecological data and each sampling sites were georeferenced using a hand-held Global Positioning System (GPS) device.

3.2.1 Adult mosquito collection

The adult mosquitoes were collected using the BG-Sentinel traps and hoover traps. These devices were used both day and evening's hours for sampling for three consecutive days in each ward. The Hoover trap captures both flying and resting mosquitoes both indoors and outdoors while the BG was fixed in the evening hours outdoor in areas perceived to have mosquitoes.

A



B

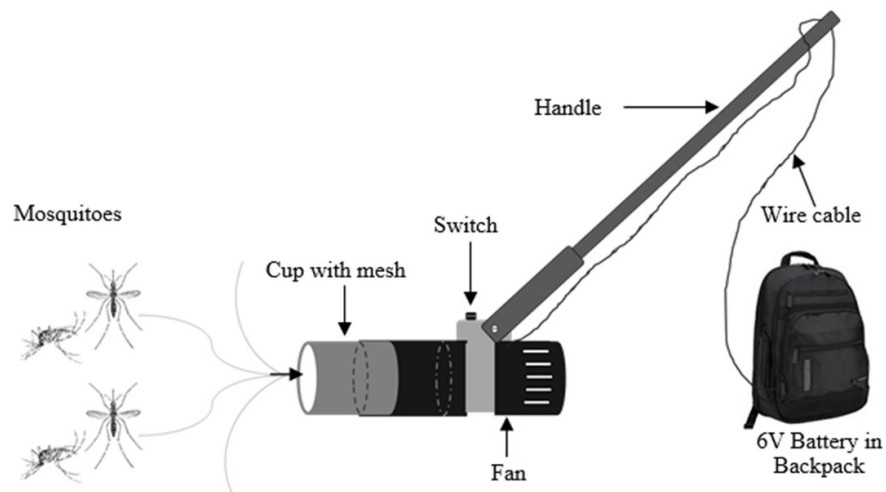


Figure 8: Mosquito traps used in the present study.

(A) A picture of BG trap and (B) A schematic drawing of hoover trap

3.2.2 Mosquito larvae collection and rearing

Mosquito larvae were captured from different locations using the standard dipping technique. At every site, all water-holding containers found were examined for the presence of larvae. Stagnant water collected in discarded articles such as car tyres, discarded tins, broken earthenware, plastic bottles and ponds were searched for larvae. The larvae were collected using large-mouth pipettes and a standard plastic dipper. The larvae were collected into a small bowl, sorted out and transferred into water filled Whirl-Pak

plastic bag using Pasteur pipette. The samples were later transported to the laboratory and adults reared in larval-adult mosquito rearing chambers under the following conditions: $26\pm 1^{\circ}\text{C}$, 18:6h light:dark diurnal cycle at approximately 45% relative humidity. The larvae were fed by grounded anchovies powder locally prepared.

3.2.3 Mosquito preparation and identification

The adult and hatched mosquitoes were euthanized using chloroform. The specimens were sorted according to species using standard taxonomic and morphological identification keys previously described by Hopkins *et al.* (1952) under the stereomicroscope. According to Smith *et al.* (2009), infection rates in field-collected mosquitoes are typically very low; therefore mosquitoes should be pooled before being homogenized. The mosquitoes were pooled into groups of 15 to 25 mosquitoes according to their sex, species, whether fed or unfed, and location. The mosquitoes were preserved in RNA later® (Ambion®, Inc., Texas, US) and kept in the freezer (-80°C) for later screening of MBV using (RT-PCR). Some mosquitoes were kept dry (without RNA later®) in labelled sterile screw top cryovial tubes (hole created on top) under room temperature. The adult mosquitoes (male and female) that emerged from larvae were pooled together.

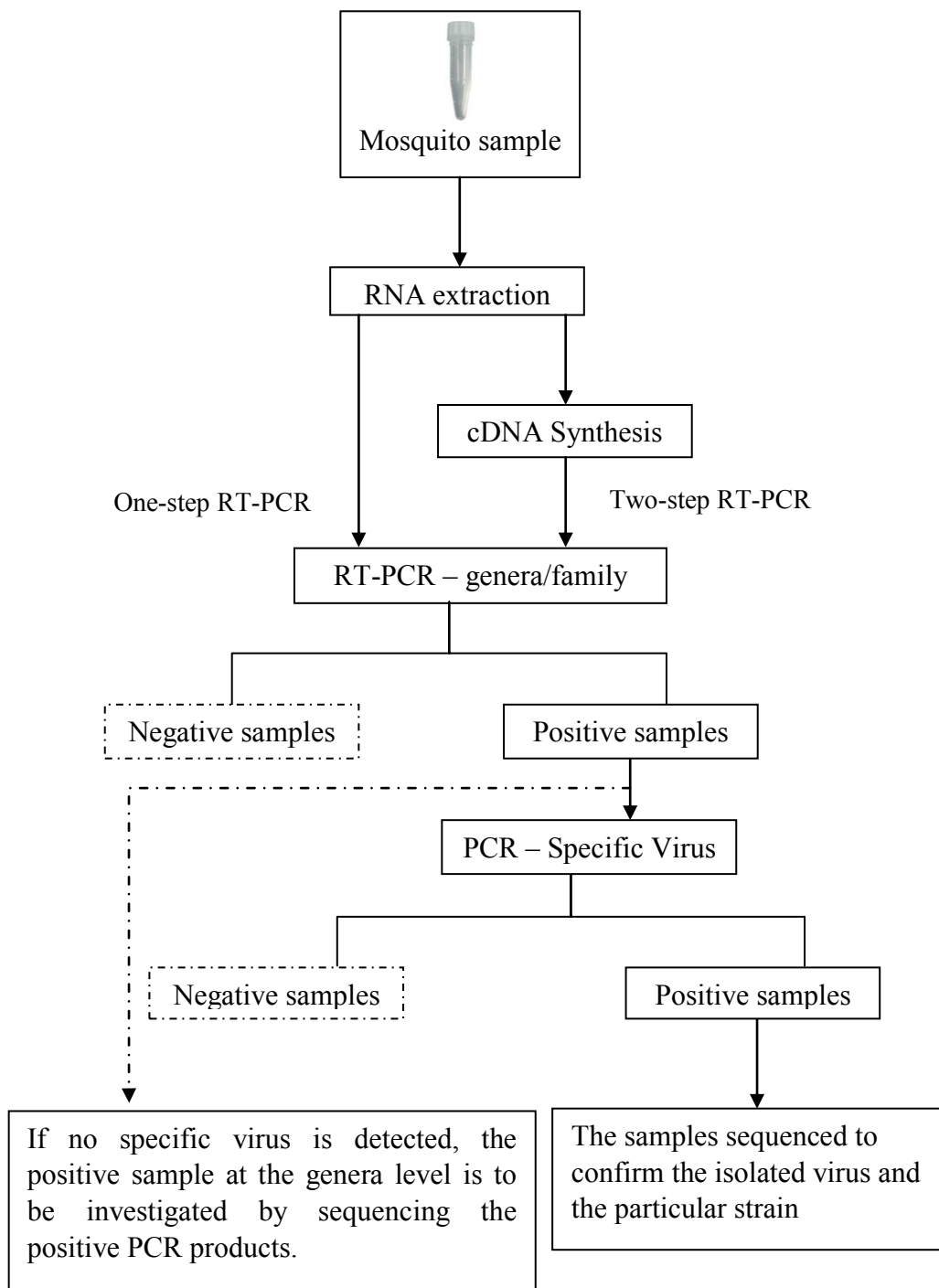


Figure 9: Flow Chart of Detection of Mosquito-borne Virus

3.3 Molecular Detection of Mosquito-borne Virus

The flow chart of the molecular detection of mosquito-borne viruses is described in Fig. 9.

3.3.1 Viral RNA extraction from mosquitoes

Total RNA was extracted using Viral RNA Mini Kit (Qiagen, Helden, Germany) according to the manufacturer's instructions with little modification (Appendix I). Briefly, phosphate-buffered saline (PBS) was added to the whole-mosquito tissue, homogenized using a plastic application stick. The samples were then lysed using lysing buffer followed by addition of ethanol to precipitate proteins. The lysate was passed through a spin column and washed with washing buffers. The RNA was later eluted from the columns with 60 μ L of RNase free water. The extracted RNA was stored at - 40 °C until RT-PCR.

3.3.2 Complementary DNA (cDNA) Synthesis - (Two – Step)

The complementary DNA (cDNA) synthesis was performed using SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufactures instruction.

Table 3: cDNA Synthesis Mix I for a single sample

No.	Component	Amount (μ L)
1	Random Hexamer	1
2	dNTPs	1
	Total Volume per reaction	2

The mixture in (Table 3) was aliquoted into a PCR tube and 8 μ L of RNA template was added. This was incubated at 65 °C for five minutes to denature, then cooled to 4 °C for five minutes. The cDNA synthesis mix II was prepared (Table 4).

Table 4: cDNA Synthesis Mix II for a single sample

No	Component	Amount (μL)
1	10X RT Buffer	2
2	25mM MgCl_2	4
3	0.1M DDT	2
4	RNase OUT Inhibitor	1
5	Super Script® III reverse transcriptase	1
	Total Volume per reaction	10

To the PCR tubes 10 μL of cDNA synthesis mix (Table 4) was added and mixed and incubated in the thermocycler at the following conditions: 25 °C for 5 minutes, 50 °C for 50 minutes terminate the reaction at 85 °C for 5 minutes and 4 °C hold temperature. 1 μL of RNase H was added to each PCR tube and incubated in the thermocycler for 20 minutes at 37 °C and 10 °C hold temperature. The final volume for this reaction was 21 μL . The cDNA was stored at -20 °C and was used for PCR.

3.3.3 Viral detection by PCR

The viruses were screened by PCR using the cDNA and primers targeting the virus genera – *Flavivirus* and *Alphavirus*; and the family *Bunyavirus* as shown in (Table 5). For samples, which tested positive at the family or genera level, further test was done with specific primers (Table 6) that target conserved genes in the specific virus belonging to these family and genera.

Table 5: Primers used for the screening of mosquito-borne virus family/genera

Virus	Target	Primer	Primer Sequence	Amplicon	References
	Gene/Protein	Name	(5' →3')	Size (bp)	
Alphavirus	nsP4	VIR 2052F	TGG CGC TAT GAT GAA ATC TGG AAT GTT	150	Eshoo <i>et al.</i> , (2007)
		VIR 2052R	TAC GAT GTT GTC GTC GCC GAT GAA		
Bunyavirus	Nucleocapsid	BCS82C	ATG ACT GAG TTG GAG TTT CAT GAT GTC GC	251	Kuno <i>et al.</i> , (1996)
	Protein	BCS332V	TGT TCC TGT TGC CAG GAA AAT		
Flavivirus	NS5	FU 1	TAC AAC ATG ATG GGA AAG AGA GAG AA	220	Kuno <i>et al.</i> , (1998)
		CFD2	GTG TCC CAG CCG GCG GTG TCA TCA GC		
18S rRNA	18S rRNA	18S417	ACGGGGAGGTAGTGACGAGAAATA	504	Hoffmann <i>et al.</i> , (2004)
	18S rRNA	18S920c	TAATACTAATGCCCCCAACTACTT		

Table 6: Primers pairs used for detection of specific MBV

Virus	Target	Primer	Primer Sequence	Amplicon	References
	Gene/Protein	Name	(5'→ 3')	Size (bp)	
Chikungunya	5NTR	CHIK3F	CACACGTAGCCTACCAGTTTC	98	Smith <i>et al.</i> (2009)
		CHIK3R	GCTGTCAGCGTCTATGTCCAC		
Dengue	Structural	D1	TCA ATA TGC TGA AAC GCG CGA GAA ACC G	511	Lanciotti <i>et al.</i> (1992)
	polyprotein	D2	TTG CAC CAA CAG TCA ATG TCT TCA GGT TC		
Rift Valley	Glycoprotein M	RVF1	GAC TAC CAG TCA GCT CAT TAC C	551	Kifaro <i>et al.</i> (2014)
Fever	gene	RVF2	TGT GAA CAA TAG GCA TTG G		

3.3.4 One-step RT-PCR

Roche TITAN One RT-PCR Kit was used and the master mixes were made according to manufacturer's instructions. To the PCR tubes 8.5 μ L of master mix I (Table 7) were aliquoted and 4 μ L of the RNA template was added. 12.5 μ L of the master mix II was added to make a total volume of 25 μ L.

Table 7: One-step RT-PCR master mixes for a single sample

No	Component	Amount (μ L)
Master mix I		
1	0.2 mM dNTP	0.75
2	5 mM DTT	1.25
3	5U RNase Inhibitor	0.5
4	10 μ m Primer (each)	0.5
5	PCR grade H ₂ O	0.5
6	RNA extracts	4
Master mix II		
7	PCR Grade H ₂ O	7
8	5x RT-PCR buffer	5
8	Enzyme mix	0.5
Total Volume per reaction		25

The following cycling conditions were used for a one-step reverse transcription at 50 °C for 30 min followed by 94 °C for 2 minutes. 40 cycles of thermo-cycling were then performed at 94 °C for 30 seconds, 48 °C – 54 °C for 30 seconds, 68 °C for 2 minutes for 7 cycles, then the remaining 33 cycles at 94 °C for 30 sec, 55 °C for 30 seconds, 68 °C for 2 minutes and 1 cycle of 68 °C for 7 minutes.

3.3.4.1 PCR conditions for Arbovirus detection

Table 8: PCR master mix for arbovirus detection

No	Component	Amount (μL)
1	Enzyme mix Buffer	12.5
2	Forward primer	0.5
3	Reverse primer	0.5
4	RNase free water	10.5
5	cDNA sample	1
Total Volume per reaction		25

The PCR amplification condition steps is as follows 95 °C for one minute, followed by a 35 cycles of denaturation at 95 °C for 30 seconds, primer-annealing temperature at 55 °C for 50 seconds, and primer extension at 72 °C for 30 seconds. The reaction was then subjected to final extension step at 72 °C for 10 minutes and then cooled at 4 °C. All PCR amplifications were carried out at 25 μL volumes per tube. Thermal profiles were performed in a GeneAmp® PCR system 9700 (Applied Biosystems, USA).

3.3.5 Agarose gel electrophoresis

Gel electrophoresis of the amplicon was performed using 1% agarose gel in 0.5X Tris-Borate EDTA buffer and stained with Gel-Red nucleic acid stain (Phenix Research Products, Candler, USA). Each gel-well was loaded with 5 μL of the PCR product and 1 μL blue 6X DNA loading dye (Promega, Madison, USA) at 80 volts for 40 minutes. The bands were visualised by UV fluorescence light using a gel photo imaging system (EZ Gel Doc, Bio Rad, USA) to capture DNA embedded in the gel.

3.4 Sanger Sequencing

Some flavivirus suspected PCR product were selected and purified using GenElute™ PCR Clean-Up Kit – (Sigma-Aldrich) (Appendix II). High-throughput Sanger sequencing was outsourced and performed using ABI-PRISM 3130 Genetic Analyzer (Applied

Biosystems, Foster City, CA). The basic principle of Sanger sequencing is that it synthesizes DNA strands that are complementary to a template DNA strand. The sequencing reaction uses normal deoxynucleoside triphosphates (dNTPs) and modified dideoxynucleoside triphosphates (ddNTPs) for strand elongation. The ddNTPs are chemically altered with a fluorescent label and with a chemical group that inhibits phosphodiester bond formation, causing DNA polymerase to stop DNA extension whenever a ddNTP is incorporated. The resulting DNA fragments are subjected to capillary electrophoresis, where the fragments flow through a gel-like matrix at different speeds according to their size. Each of the four modified ddNTPs carries a distinct fluorescent label. The emitted fluorescence signal from each excited fluorescent dye determines the identity of the nucleotide in the original DNA template (Sanger *et al.*, 1977).

Geneious software versions 5.3.6 was used to organize and analyze the sequence data. The Basic Local Alignment Search Tool (BLAST) was used to find regions of similarity of the sequence in the gene bank.

3.5 Statistical Data Analysis

Simpson's Index (D) and Simpson's Index of Diversity (1-D) was calculated using the formula below to determine mosquito species diversity.

$$D = \frac{\sum n(n-1)}{N(N-1)}$$

Where; n = the total number of mosquitoes of a particular species, and N = the total number of mosquitoes of all species in each collection (Simpson, 1949).

Excel was used to analyse the proportion of mosquito by genus and species, and the proportion of positive pools for arbovirus, and to calculate the Simpson's Index (D).

CHAPTER FOUR

4.0 RESULTS

4.1 Mosquito Abundance

A total of 7649 mosquitoes were collected 7224 were collected as adults and 425 reared to adult larvae and pupa. They were collected in Morogoro municipality from the five wards namely, Kiwanja cha Ndege, Mbuyuni, Mwembesongo Mazimbu and Kilakala. More mosquitoes were collected from Mbuyuni representing 43.7% (n =3156), followed with Kiwanja cha Ndege (22%; n = 1587), Mwembesongo (19.2%; n = 1387), Mazimbu (7.6%; n = 549) and Kilakala (7.5%; n = 545) (Table 10). Five mosquito genera (*Aedes*, *Anopheles*, *Culex*, *Eretmapodites* and *Mansonia*) were identified in the collections, but not all these five genera were identified in a single location. Although not all mosquito species could probably be sampled in the sampling areas due to time and labour constraints, equal sampling efforts were considered in sampling.

As shown in (Table 9 and 10), the *Culex* genus recorded the highest number of mosquitoes sampled (54.5%; n = 3939) from all the sites in which over half (53.9%; n = 3891) was *Culex quinquefasciatus*, as the most frequently captured species (Fig. 10). The rest of the six *Culex* species recorded $\leq 0.2\%$. The *Aedes* genus sampled represented (45.1%; n = 3257) from all the sites with *Aedes aegypti* (44.2%; n = 3192), *Aedes pempaensis* (0.9%; n = 62) and only three *Aedes simpsoni* sampled. The genera with the least mosquitoes collected were *Anopheles*, *Eretmapodites* and *Mansonia* representing $\leq 0.2\%$. The *Anopheles* genus has two species, *An. coustani* and *An. gambiae* with $\leq 0.1\%$ whereas of the genera *Eretmapodites* and *Mansonia*, *Eretmapodites quinquevittatus* and *Mansonia africanus* with (0.2%; n = 12) and (0%; n = 2), respectively.

4.1.1 Species richness

Species richness is reported as the number of mosquito species at each location. In this study, a total of 14 species belonging to five mosquito genera of the *Culicidae* family were morphologically identified. Among members of the genus *Aedes* collected were *Ae. aegypti*, *Ae. pembaensis* and *Ae. simpsoni*. Those belonging to Anopheles included *An. gambiae* and *An. coustani* and seven species including *Culex antennatus*, *Culex quinquefasciatus*, *Culex eretmapodites*, *Culex cinereus*, *Culex decens*, *Culex (lutzia) tigripes* and *Culex zombaensis* belongs to the genus *Culex*. However *Eretmapodites* and *Mansonia* genera recorded a single species each *Eretmapodites quinquevittatus* and *Mansonia africanus*, respectively (Table 9). The generic richness was predominant at Mbuyuni, which had four genera with 12 different species. Kiwanja cha Ndege, and Mwembesongo also recorded four genera with eight and five different species, respectively. Mazimbu and Kilakala however had two genera each with four and three different species, respectively (Table 9).

Table 9: Number (%) of mosquito species collected and the Simpson's Index of Diversity

Species	Sampling Locations (wards)					
	Kiwanja cha Ndege	Mbuyuni	Mwembesongo	Mazimbu	Kilakala	Total
	No (%)	No (%)	No (%)	No (%)	No (%)	No (%)
<i>Aedes aegypti</i>	1025 (64.6)	730 (23.1)	1033 (74.5)	233 (42.4)	171 (31.4)	3192 (44.2)
<i>Aedes pemaensis</i>	6 (0.4)	44 (1.4)	4 (0.3)	6 (1.1)	2 (0.4)	62 (0.9)
<i>Aedes simpsoni</i>	0	3 (0.1)	0	0	0	3 (0.0)
<i>Anopheles coustani</i>	0	4 (0.1)	0	0	0	4 (0.1)
<i>Anopheles gambiae</i>	2 (0.1)	4 (0.1)	4 (0.3)	0	0	10 (0.1)
<i>Culex antennatus</i>	0	11(0.3)	0	0	0	11 (0.2)
<i>Culex cinereus</i>	8 (0.5)	0	0	5 (0.9)	0	13 (0.2)
<i>Culex decens</i>	0	4 (0.1)	0	0	0	4 (0.1)
<i>Culex eretmapodites</i>	1 (0.1)	5 (0.2)	0	0	0	6 (0.1)
<i>Culex quinquefasciatus</i>	533 (33.6)	2342 (74.2)	339 (24.4)	305 (55.6)	372 (68.3)	3891 (53.9)
<i>Culex tigripes</i>	7 (0.4)	3 (0.1)	0	0	0	10 (0.1)
<i>Culex zombaensis</i>	0	4 (0.1)	0	0	0	4 (0.1)
<i>Eretmapodites quinquevittatus</i>	5 (0.3)	0	7 (0.5)	0	0	12 (0.2)
<i>Mansonia africanus</i>	0	2 (0.1)	0	0	0	2 (0.0)
Total	1587	3156	1387	549	545	7224
D*	0.53	0.60	0.61	0.49	0.56	
1 - D	0.47	0.40	0.39	0.51	0.44	

D*= Simpson's Index of Diversity (D).

Table 10: Mosquito distribution (%) by genus between the sampling locations (wards).

Sampling Wards	Geographical Coordinates	<i>Aedes</i>	<i>Anopheles</i>	<i>Culex</i>	<i>Eretmapodites</i>	<i>Mansonia</i>	Total
		N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
vanja cha Ndege	S06.8220/E037.6606	1031 (31.7)	2 (14.3)	549 (13.9)	5 (41.7)	0	1587 (22.0)
uyuni	S06.8499/E037.6585	777 (23.9)	8 (57.1)	2369 (60.1)	0	2 (100)	3156 (43.7)
vembesongo	S06.8055/E037.6650	1037 (31.8)	4 (28.6)	339 (8.6)	7 (58.3)	0	1387 (19.2)
zimbu	S06.8053/E037.6581	239 (7.3)	0	310 (7.9)	0	0	549 (7.6)
akala	S06.8220/E037.6802	173 (5.3)	0	372 (9.4)	0	0	545 (7.5)
tal		3257 (45.1)	14 (0.2)	3939 (54.5)	12 (0.2)	2 (0.0)	7224

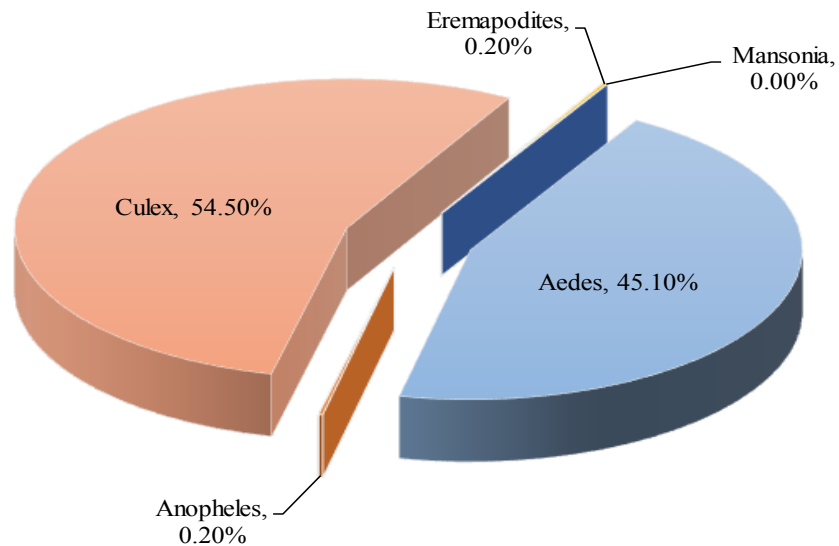


Figure 10: The proportion of mosquitoes by genus sampled from the wards

4.1.2 Species diversity

Species diversity was estimated for each location by calculating the Simpson Index (D) (Simpson, 1949) as used in a similar study (Mutebi *et al*

., 2012). This index accounts for species richness and the relative abundance of each species (Table 9). The value of D ranges between zero and one whereas an index of zero represents vast diversity and one, representing no diversity. Thus, the bigger the value of D, the lower the diversity. However, the Simpson's Index of Diversity (1-D), interpreted as the greater the index, the greater the sample diversity and vice versa (Simpson, 1949).

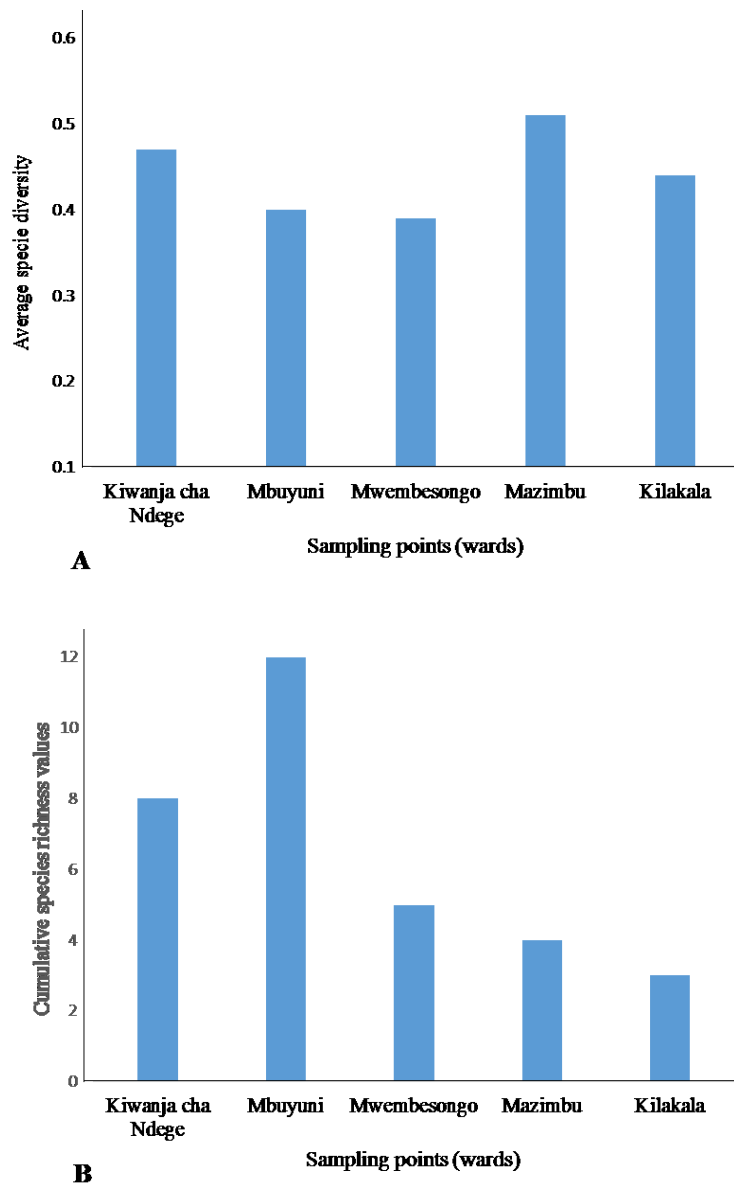


Figure 11: Simpson's Index of diversity (A) and specie richness (B) of mosquitoes of Morogoro Municipality.

Mazimbu had greater species diversity compared with the rest of the wards. However, Mbuyuni had more species richness than Kiwanja cha Ndege. Kilakala and Kiwanja cha Ndege recorded a moderate diversity as compared to Mbuyuni and Mwembesongo wards. Kilakala had less specie richness (Fig. 11).

Table 11: Number (%) and species of mosquito larvae and pupa collected within Morogoro municipality.

Number (%) and species of mosquito larvae and pupa					
Ward	<i>Aedes aegypti</i>	<i>Anopheles gambiae</i>	<i>Culex quinquefasciatus</i>	<i>Culex eretmapodites</i>	Total
Kiwanja cha Ndege	55(48.7%)	16(14.1%)	33(29.2%)	9(8%)	113 (26.1%)
Mbuyuni	25 (26.0%)	12 (12.5%)	47 (49.0%)	12 (12.5%)	96 (22.2%)
Mwembesongo	45 (46.4%)	10 (10.3%)	33 (34.0%)	9 (9.3%)	97 (22.4%)
Mazimbu	39 (54.9%)	7 (9.9%)	25 (35.2%)	0 (0.0%)	71 (16.4%)
Kilakala	26 (46.4%)	5 (8.9%)	23 (41.1%)	2 (3.6%)	56 (12.9%)
Total	190 (43.9%)	50 (11.5%)	161 (37.2%)	32 (7.4%)	433

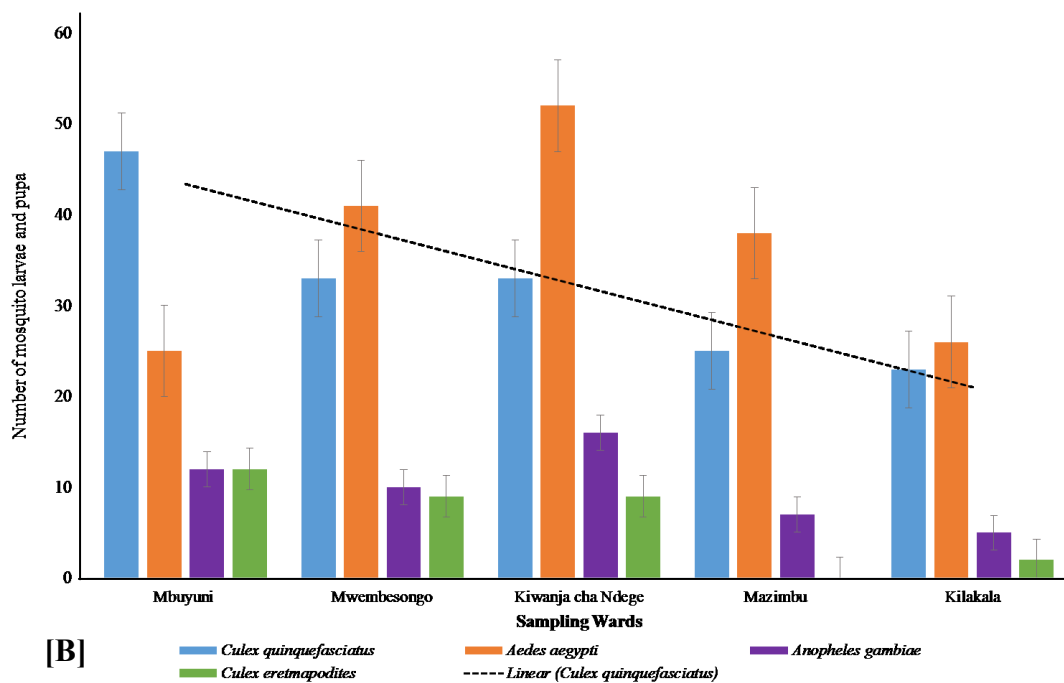
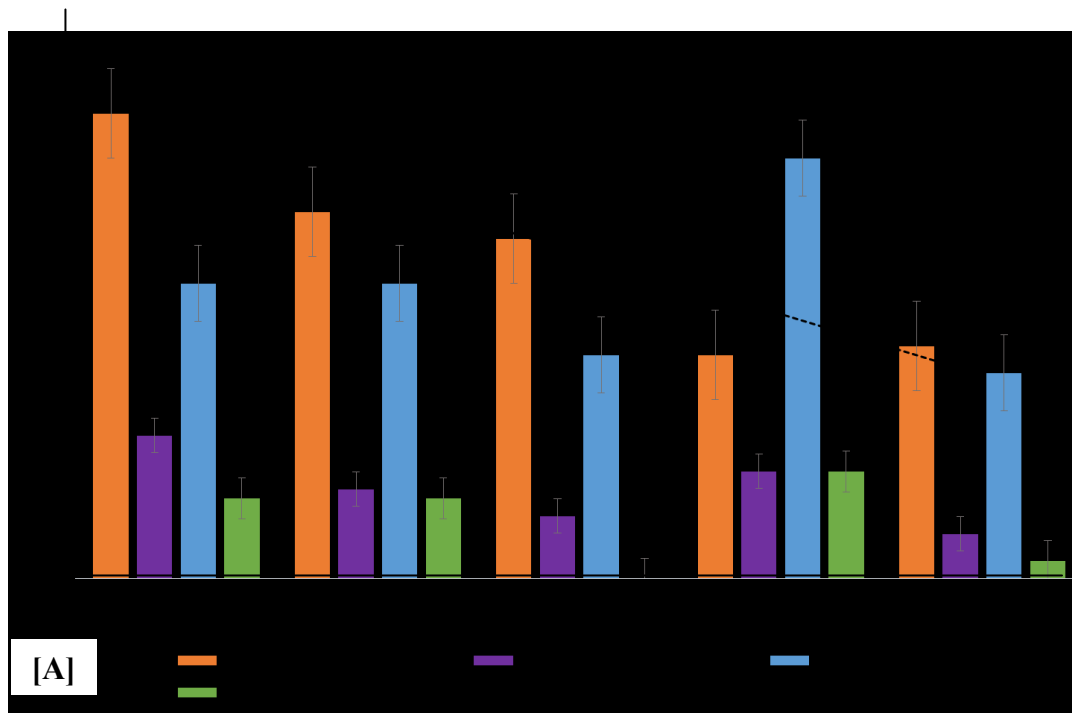


Figure 12: Average number of the different species of mosquito larvae/pupa.

Sample per each ward showing the linear increasing or decreasing order of prevalence of *Aedes aegypti* and *Culex quinquefasciatus* in A and B, respectively.

Table 12: The habitats of adult mosquitoes and larvae/pupa in Morogoro municipality

Types of habitat	Mosquitoes identified	Wards
Adult Mosquitoes		
Maize and pea plantation	<i>Culex quinquefasciatus</i> , <i>Culex eretmapodites</i>	Kiwanja cha Ndege, Mwembesongo, Mbuyuni
Grass, Lawn	<i>Aedes aegypti</i> , <i>Culex quinquefasciatus</i>	Mwembesongo, Mazimbu
Tyres	<i>Aedes aegypti</i> , <i>Culex quinquefasciatus</i>	Mbuyuni, Kilakala, Kiwanja cha Ndege, Mwembesongo, Mazimbu
Bush	<i>Culex quinquefasciatus</i> , <i>Culex eretmapodites</i> , <i>Anopheles gambiae</i>	Mbuyuni
Mosquito Larvae		
Plastic and Tin containers	<i>Culex quinquefasciatus</i> , <i>Culex eretmapodites</i> <i>Aedes aegypti</i>	Kiwanja cha Ndege, Mbuyuni
Tyres	<i>Aedes aegypti</i> , <i>Culex quinquefasciatus</i>	Mbuyuni, Kilakala, Kiwanja cha Ndege, Mwembesongo, Mazimbu
Wells	<i>Aedes aegypti</i> , <i>Culex quinquefasciatus</i>	Mwembesongo, Mazimbu



Figure 13: Habitats for adult and larvae mosquitoes.

The adults were found at (A) plantain/banana plantation (B) mixed-farms with car tyres; and the larvae /pupa collected from (C) old car tyres, and (D) concrete wells.

4.2 Arboviral Screening

The female *Aedes aegypti* mosquitoes collected were screened for viruses and of 23 pools, 52.17% (n=19) were suspected flavivirus positives shown in (Fig. 14) below. Nonetheless, same pools were negative for *Bunyavirus* and *Alphavirus*.

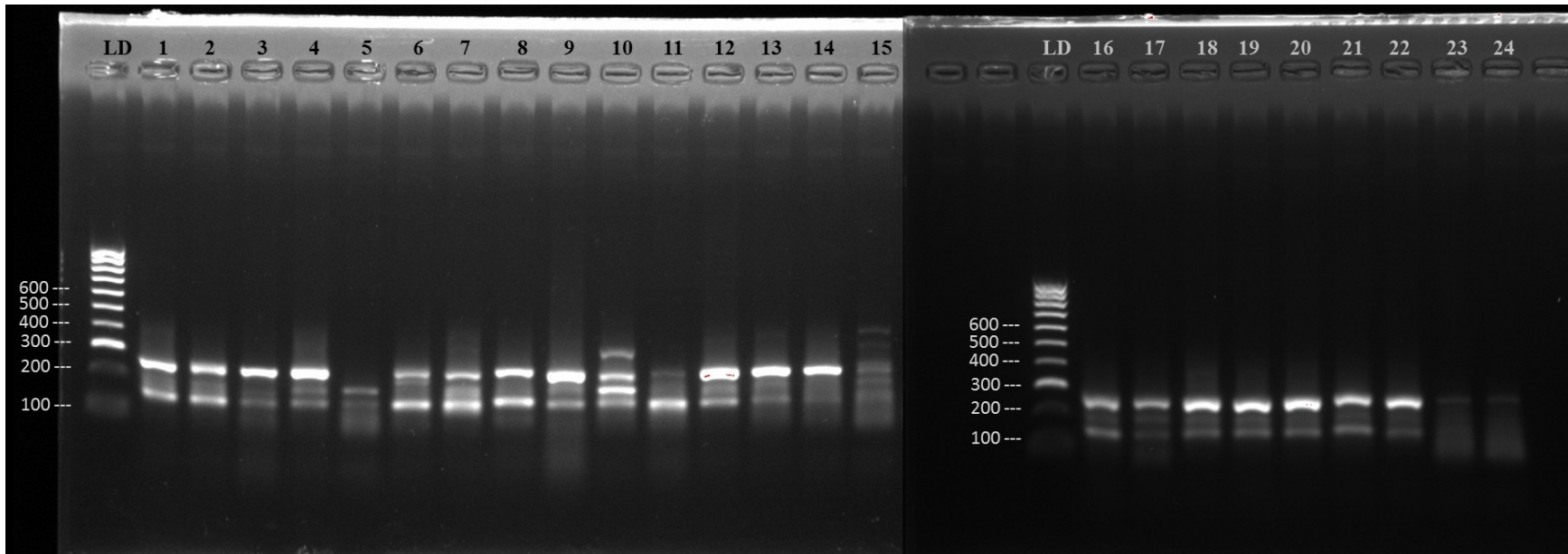


Figure 14: Detection of flavivirus genome in mosquitoes.

Detection was done using RT-PCR in 23 pools of *Aedes aegypti* using these primers: FU1 (forward) and CFD2 (reverse) to target the NS5 gene. The expected PCR product size is 220bp. LD-DNA maker, F-female; lane 18, 19, 22, 23 are males; 12, 13, 20, 21 are adults breed from larvae; 24 is cDNA control; the rest lanes are females

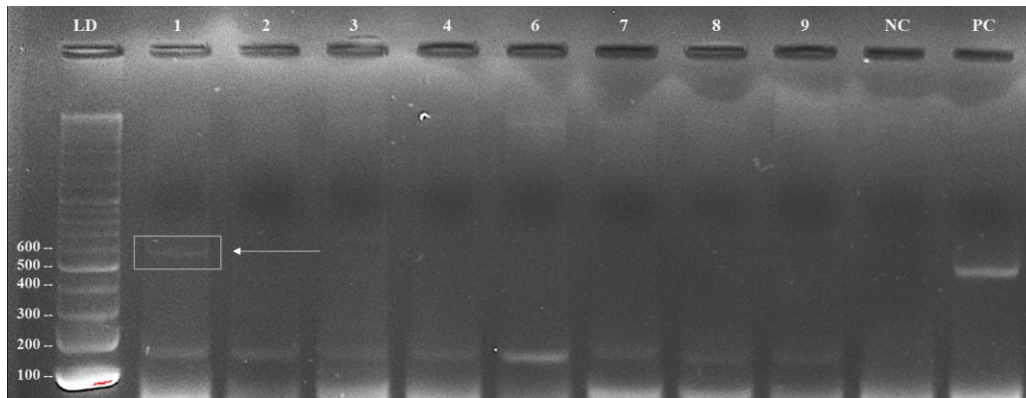


Figure 15: Detection of dengue virus in mosquitoes.

The DENV primers D1 and D2 were used to screen dengue virus in mosquitoes using RT-PCR to screen samples that were flavivirus positive. The expected PCR product size is 511 bp. Lane 1 shows a suspected dengue virus (DENV) and the rest shows negative. LD- DNA maker, NC- negative control; PC - DENV positive control.

The suspected band (Fig. 15) was purified from the gel using QIAquick gel purification kit (according to manufacturers' instruction, Appendix II). PCR and gel electrophoresis was performed on the purified DNA but the result proved negative (Fig. 16).

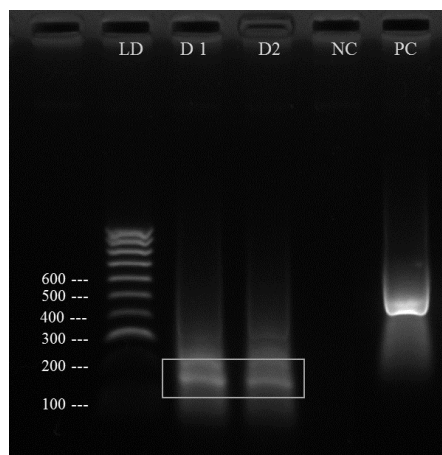


Figure 16: RT-PCR product of the purified suspected DENV.

The expected band size is 511 bp. D1 and D2 are duplicates of the suspected sample of about 170 bp. NC and PC are negative and positive control, respectively.

To determine the integrity of RNA extracted, RT-PCR was performed on 15 mosquito RNA samples using the Roche TITAN One Tube RT-PCR kit (Roche, Germany) with mosquito 18S rRNA primers.

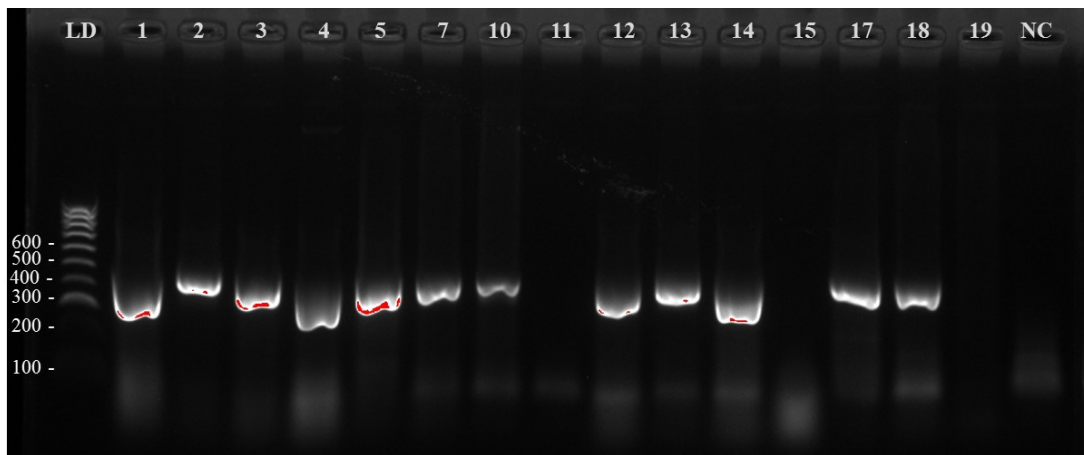


Figure 17: RT-PCR amplification of mosquitos' 18S rRNA sequence.

Amplification of 18S rRNA sequence of *Aedes aegypti* were amplified using 18S417 and 18S920c primers to determine the integrity of the RNA extracted. NC lane is the negative control whereas lanes 11 and 15 were negative, and the rest shows positive

Table 13: The BLAST result of suspected positive flavivirus

	BLAST result	Percentage Match	Base Pair Identities	NCBI Accession Number
1	<i>Aedes aegypti</i> AAEL017001-RA partial mRNA	85%	159/186	XM_011494734
2	Kamiti River virus from <i>Aedes aegypti</i> NS5 gene, partial cds	94%	107/114	DQ335465
3	<i>Culex chidesteri</i> partial 18S rRNA gene	99%	97/98	HE600016
4	<i>Culex quinquefasciatus</i> hypothetical protein, mRNA	99%	97/98	XM_001849752
5	Flavivirus CbaAr4001 NS5 gene, partial cds	100%	87/87	DQ232622
6	Phlebotomus flavivirus Alg_F8 NS5 gene, partial cds	99%	99/100	FJ817076
7	<i>Aedes</i> flavivirus strain AEFV-SPFLD-MO-2011-MP6, complete genome	79%	95/120	KC181923
8	Yellow fever virus strain Angola71, complete genome	100%	28/28	AY968064
9	Tick-borne encephalitis virus clone Vs[Hypr_prM-E], complete genome	75%	99/132	KP716972
10	Dengue virus isolate 010DENV_PEBR15 polyprotein gene, partial cds	100%	38/38	KU232287

CHAPTER FIVE

5.0 Discussion

The re-emergence and the rate of unexpected spread of MBV in recent times is alarming. A number of factors in the globalized world, such as competent vectors, increasing and rapid transportation of people and animals favour the worldwide spread of the viruses (Weissenbock *et al.*, 2010). The devastating nature of the viruses require surveillance as part of an early warning system that could provide information for the identification of potential risks and to provide appropriate prevention and control measures to curb this menace. In recent field-based studies in parts of Tanzania, there have been reports on the role of mosquito-borne viral antibodies within humans, livestock, and wildlife populations which demonstrate that these viruses are in circulation (Ochieng *et al.*, 2013; Sumaye *et al.*, 2015). The present study however assessed the abundance and diversity of mosquito species, and the viral infectivity in the vector population in Morogoro municipality.

This study reports presence of substantial potential arboviral vectors. Most of the species sampled belongs to these five genera of mosquito namely *Aedes*, *Anopheles*, *Culex*, *Eretmapodites* and *Mansonia*. Mazimbu has the greater species diversity compared with the rest of the wards, whereas Mbuyuni has more species richness than Kiwanja cha Ndege and Kilakala, the least. However, Kilakala and Kiwanja cha Ndege recorded a moderate diversity as compared to Mbuyuni and Mwembesongo wards. This result can be attributed to the vegetation covers and human activities such as, improper disposal of containers, and the presence of old tyres as it harbour stagnant water that enhances the breeding and the spread of mosquito species. The most predominant species sampled were *Culex quinquefasciatus* and *Aedes aegypti* reported in other findings (Mweya *et al.*, 2015; Mboera *et al.*, 2016) as most common species and their probable roles in transmission or

the maintenance of these viruses. In about 85% (n=12) of N=14 of the mosquitoes collected, MBV of medical and veterinary importance have previously been isolated. The major arboviral epidemic vector species include *Aedes aegypti*, the principal vector of DENV (Mboera *et al.*, 2016); CHIKV (Jupp and McIntosh, 1988) and Zika virus (ZIKV) (Marchette *et al.*, 1969). A comprehensive list of arboviruses that have been isolated from mosquito species elsewhere has been published by (Mutebi *et al.*, 2012). The presence of these vectors shows a high potential for maintenance and transmission of arboviruses in the Morogoro municipality.

Although vector distribution is an important factor in virus transmission, other factors also contribute to the disease distribution (Herrera *et al.*, 2006). These include virus presence, suitable breeding sites and sources of blood meal (Heinrich *et al.*, 2012). It is observed that the distribution of vectors was restricted to certain areas probably due to some ecological and environmental adaptations. In this study, car tyres, clogged gutters, short concrete wells and containers that collected water were found to be the breeding grounds. This has been reported in other studies (Mweya *et al.*, 2015; Mboera *et al.*, 2016) as susceptible in harbouring larvae particularly those of *Aedes spp.* This was evident with the high presence of mosquito species captured, particularly the *Aedes spp.* and *Culex spp.* Similar findings by Mboera *et al.* (2016) has reported same of outdoor breeding mosquitoes in rural areas of Tanzania. The findings however demonstrated presence of high larval infestation, which supported the high numbers of *Aedes aegypti* collected particularly in Kiwanja cha Ndege, Mwembesongo and Mazimbu wards evidenced the increased number of garages with used old tyres. This suggests high probability of any eventual transmission in the municipality, particularly in the Kiwanja cha Ndege ward with the highest larvae, should there be any introduction of arbovirus.

The proliferation of garages and the improper disposal of car tyres create a favourable environment for mosquitoes to breed as these tyres collect water during raining seasons. There have been reports that *Aedes albopictus*, which originated from Asia is currently the most invasive mosquito in the world, as they vector many viruses, including DENV, WNV and La Crosse virus (Gratz, 2004; Benedict *et al.*, 2007). They breed mainly in used car tyres and their invasion into new areas is often initiated through the transportation of mosquito eggs via international trades of used tyres (Gratz, 2004). It is suggested that if no proper methods of car tyres disposal are implemented, the vector *Aedes albopictus* that has not yet been reported in Tanzania could become established.

Although *Aedes spp* were predominantly found in tyres, it is worth noting that at Sokoine University of Agriculture (SUA) campus, in the Mbuyuni ward, where there were no garages, *Aedes aegypti* was prevalent particularly in the veterinary faculty. This can be attributed to the presence of some animals e.g. horses, goats and dogs kept in the area, as well as clogged drainage channel that provide the breeding grounds for these mosquitoes. Kweka *et al.* (2011) point this to the role of animal urine in the oviposition site preference of culicine mosquitoes, stating that host-seeking mosquitoes respond to carbon dioxide (CO₂), ammonia from urine that emanates from vertebrates. Mosquito's reproductive success also depends largely on its ability to search for suitable oviposition site to ensure survival for its progeny (Smallegange *et al.*, 2011). This implies that communities that house animals or keep livestock ranches are highly at risk as these vectors could be prevalent in these areas due to the factors aforementioned.

There are several reports of an isolation of WNV from *Culex quinquefasciatus*. This specie was reported to be the cause of the widespread of WNV in neighbouring Kenya (LaBeaud *et al.*, 2011) and birds in New York State (Bernard *et al.*, 2001) which poses a

public health threat. Wild birds are reported to be the main reservoir hosts of WNV (Reisen *et al.*, 1990). Although these species are ornithophilic, it also feeds readily on mammals including humans (Reisen *et al.*, 1990) hence the potential risk of transmitting WNV to humans. It is alarming as *Culex quinquefasciatus* the most prevalent caught in all the sampling sites in the municipality. This indicates that the species has adapted to the human and animal habitation. Blocked drainage channels, containers and wading pools are facilitating their breeding. It is therefore necessary to consider field surveillance in birds as well for the arboviral activity.

Habitat characteristics and its proximity to densely populated areas creates blood meal source for reproduction and perhaps effective transmission of viruses. Arguably, the greatest public health threat of virus transmission is the potential emergence in urban areas in which dense populations of humans and vectors exist. Worth noting is the fact that some garage owners cohabit with the livestock in their yards. In fact, it was observed that most of the female mosquitoes sampled in these areas were blood fed, an obvious reason that there was available source of blood meal. It is important to note that significant proportion of mosquitoes collected in all these areas are potential vectors for DENV, CHIK, RVFV as corroborated by other studies elsewhere (Mboera *et al.*, 2016; Mutebi *et al.*, 2012; Mweya *et al.*, 2015), an indication that human-vector interaction is on the increase. These findings provide an important step to identify potential areas that might influence host-vector interactions and ultimately the emergence of mosquito-borne virus.

Despite the established serological evidence of RVFV in livestock (Wensman *et al.*, 2015), DENV and CHKV in humans (Chipwaza *et al.*, 2014) in the Morogoro region, this study was unable to demonstrate arbovirus activity in the mosquitoes collected. Those viruses detected in the aforementioned studies were thought to be as a result of viral

spillovers from the wild animals in the Mikumi National park into the human population. The absence of the virus in the samples could be as a result of possible low rate of the virus in circulation, or that sampling was done at a period where there was no active outbreak and the locations may also have affected the outcome. However, the presence of arbovirus is gaining grounds and can easily go unnoticed particularly in malaria endemic areas. This is because most febrile illnesses including MBV mimic the symptoms of malaria, as they share similarities in clinical manifestations. There has been arbovirus co-infection in suspected febrile malaria and typhoid patients reported in Nigeria (Baba *et al.*, 2013) and concurrent malaria and arbovirus infections in Senegal (Sow *et al.*, 2016). It is important to have periodic surveillance programmes in monitoring the viral activity in the mosquito vectors, as well as animals and the human populations (Hall *et al.*, 2012) in the municipality to enhance our understanding of their role.

Although only 23 pools of mosquitoes were tested for arboviruses, this number is significant for any virus to be detected (Jupp *et al.*, 2000). It has been reported that just a fraction or pools of mosquito population collected, can yield suitable results, and by that it can also conserve time and resources (Jupp *et al.*, 2000). Chengula *et al.* (2013) reported the difficulty in detecting viruses in mosquitoes during inter epizootic period (IEP) in Tanzania despite detection of viral antibodies among livestock and wildlife populations. Similarly, other reports could not detect viruses in mosquitoes such as WNV (Hoffmann *et al.*, 2004) and RVF (Mweya *et al.*, 2015; Wensman *et al.*, 2015). Nonetheless, several studies have demonstrated the detection of the virus in mosquitoes in Tanzania (Ochieng *et al.*, 2013; Mboera *et al.*, 2016), in Kenya LaBeaud *et al.* (2011). The salient reason that can be attributed to this observation is that, areas where the virus was detected in the mosquitoes have been cited for previous outbreaks.

The RT-PCR is a reliable method used in arboviral screening and as such has been used successfully in the past to amplify viral groups such as alphavirus (Eshoo *et al.*, 2007), bunyavirus (Kuno *et al.*, 1996) and flavivirus (Kuno *et al.*, 1998). The structural component of eukaryotic cytoplasmic ribosomes RNA in eukaryotic cells is 18S rRNA with highly conserved regions. These regions can be amplified using the 18S417 and 18S920c primer pairs to serve as an internal control as it is essential to verify the integrity of viral RNA (Hoffmann *et al.*, 2004). These primers were used to amplify 15 samples. It was however observed that samples that were negative for 18S primers were negative for flavivirus primers. This corroborates the prior findings of Hoffman *et al.* (2004) that used 18S primers as a means to authenticate the outcome of arboviral screening. It is therefore necessary to establish the quality and the integrity of the extracted RNA from mosquito specimens.

By using the BLAST to analyze the suspected positive flavivirus sequence data, it was discovered that flavivirus genome matched and shared similarities with mosquito genome sequence, such as *Aedes* and *Culex spp.* mRNA, mosquito rRNA and some other flavivirus groups e.g. Kamiti River virus (KRV). The KRV is an insect-only flavivirus that cannot replicate in vertebrate cells matched with NS5 gene region of other flavivirus related sequences (Crochu *et al.*, 2004). The genome of *Aedes albopictus* and *Aedes aegypti* also contain these flavivirus-related sequences discovered in their dsDNA genome demonstrating integration into a eukaryotic genome of a multigenic sequence from RNA virus (Crochu *et al.*, 2004).

The suggestion of reverse transcriptase activity effectively copying viral RNA into DNA and later integrating into the host genome was made by Zhdanov (1975), and has been proven in other studies (Crochu *et al.*, 2004). This suggests that the MBV can be

transcribed and their genome sequences integrated into mosquitoes' genomes. This explains the amplification of RNA that gave rise to the many flavivirus positive fragments. For instance, the KRV sequences have been found in *Aedes albopictus* attributed to genome integration (Roiz *et al.*, 2009). KRV demonstrates the presence of an insect-only flavivirus in nature and raises questions regarding potential interactions between this virus and other MBV in competent vector populations. Future screening should consider sequencing all putative viral positive samples for verification. It is however proposed that the use of 18S rRNA primers as an internal positive control for RT-PCR remains an option for mosquito-borne viral screening studies as it minimizes the risk of obtaining false negative results (Menzel *et al.*, 2002; Hoffmann *et al.*, 2004).

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, this study has shown that arbovirus vectors are distributed and well established throughout the Morogoro municipality. The species collected have previously been documented as potential vectors. Although no specific virus was detected nonetheless, the result provides insight into the presence of these potential vectors, and their close proximity to humans pose a high risk of arbovirus transmission in the municipality. It is necessary therefore to continue to conduct assessment of arbovirus risk to the public and veterinary health in the municipality. Immediate control measures could include public education and how to deal with 'key containers' such as used car tyres and cans that hold stagnant or dirty water and create the enabling environment for breeding mosquitoes. Also, small but major isolated breeding grounds could be targeted in applying larvicide or clearing such places, as a gradual vector control management.

6.2 Recommendations

1. The high prevalence of the vector in the municipality calls for public education campaign on the medical and veterinary importance of these vectors as this could reduce the risk of exposure.
2. There is the need to enhance continuous surveillance for arbovirus activity in livestock, human and vector population across the municipality to avoid maintenance of the virus in mosquitoes that may lead to future outbreaks.
3. Integrating arboviral screening/diagnosis at point of care routine laboratory investigations should be encouraged to reduce mosquito-borne viral misdiagnosis as malaria.
4. There should be proper disposal of used car tyres to curtail the breeding grounds

for *Aedes aegypti* in particular, as well as other mosquito species.

5. Education on environmental management and manipulation to control mosquito breeding sites.
6. It is necessary to establish the quality and integrity of RNA extracted from mosquitoes before clinically drawing conclusions.
7. Future screening should consider sequencing all putative viral positive samples for the presence of insect only flaviruses e.g. KRV or otherwise.
8. There is the need to investigate the prevalence of the insect only virus e.g. KRV in the mosquitoes.

REFERENCES

- Afrane, Y. A., Lawson, B. W., Githeko, A. K. and Yan, G. (2005). Effects of microclimatic changes caused by land use and land cover on duration of gonotrophic cycles of *Anopheles gambiae* (Diptera: Culicidae) in western Kenya highlands. *Journal of Medical Entomology*, 42(6): 974–980.
- Agarwal, A., Dash, P. K., Singh, A. K., Sharma, S., Gopalan, N., Rao, P. V. L. and Reiter, P. (2014). Evidence of Experimental Vertical Transmission of Emerging Novel ECSA Genotype of Chikungunya Virus in *Aedes aegypti*. *PLoS Neglected Tropical Diseases*, 8(7): 4–9.
- Ahmad, R., Ismail, A., Saat, Z. and Lim L. H. (1997). Detection of dengue virus from field *Aedes aegypti* and *Aedes albopictus* adults and larvae. *Southeast Asian Journal of Tropical Medicine Public Health*, 28(2): 138-42.
- Alphey, L., Benedict, M., Bellini, R., Clark, G. G., Dame, D., Service, M. W. and Dobson, S. L. (2010). Sterile-Insect Methods for Control of Mosquito-Borne Diseases: An Analysis. *Vector-Borne and Zoonotic Diseases*, 10(3): 295–311.
- Alphey, L., McKemey, A., Nimmo, D., Neira O, M, Lacroix, R., Matzen, K. and Beech, C. (2013). Genetic control of *Aedes* mosquitoes. *Pathogens and Global Health*, 107(4): 170–179.
- Amusan, A. A. S., Mafiana, C. F., Idowu, A. B. and Olatunde, G. O. (2005). Sampling mosquitoes with CDC light trap in rice field and plantation communities in Ogun State, Nigeria. *Tanzania Health Research Bulletin*, 7(3): 111–116.

- Anyamba, A., Chretien, J., Small, J., Tucker, C. J., Formenty, P. B., Richardson, J. H. and Linthicum, K. J. (2009). Prediction of a Rift Valley fever outbreak. *The National Academy of Sciences, USA*, 106(3): 955–959.
- Atkins, G. J. (2013). Review: The Pathogenesis of Alphavirus. *Hindawi Publishing Corporation*, 5: 163–175.
- Baba, M., Logue, C. H., Oderinde, B., Abdulmaleek, H., Williams, J., Lewis, J. and D'Agaro, P. (2013). Evidence of arbovirus co-infection in suspected febrile malaria and typhoid patients in Nigeria. *Journal of Infection in Developing Countries*, 7(1): 51–59.
- Blakqori, G., Lowen, A .C. and Elliott, R. M. (2012). The small genome segment of Bunyamwera orthobunyavirus harbours a single transcription-termination signal. *The Journal of general virology*, 93(7): 1449–1455.
- Barrera, R., Mackay, A. J. and Amador, M. (2013). An improved trap to capture adult container-inhabiting mosquitoes. *Journal of American Mosquito Control Assoc.*, 29(4): 358–368.
- Becker, N., Petrić, D., Zgomba, M., Boase, C., Madon, M., Dahl, C. and Kaiser, A. (2010). Medical Importance of Mosquitoes. In *Mosquitoes and Their Control SE - 3*. Springer Berlin Heidelberg. pp. 25–42.
- Beerntsen, B. T. and Christensen, B. M. (2000). Genetics of Mosquito Vector Competence. *Microbiology and Molecular Biology Reviews*, 64(1): 115.

- Benedict, M. Q., Levine, R. S., Hawley, W. A. and Lounibos P. L. (2007). Spread of the Tiger: Global Risk of Invasion by the Mosquito *Aedes albopictus*. *Vector Borne Zoonotic Disease*, 7(1): 76–85.
- Bernard, K. A., Maffei, J. G., Jones, S. A., Kauffman, E. B., Ebel, G. D., DuPuis, A. P. and Kramer, L. D. (2001). Comparison of West Nile virus infection in birds and mosquitoes in New York State in 2000. *Emerging Infectious Diseases*, 7(4): 679–685.
- Bird, B. H., Khristova, M. L., Ksiazek, T. G. and Nichol, S. T. (2007). Complete genome analysis of 33 ecologically and biologically diverse Rift Valley fever virus strains reveals widespread virus movement and low genetic diversity due to recent common ancestry. *Journal of Virology*, 81(6): 2805–2816.
- Bolling, B., Weaver, S. and Vasilakis, N. (2015). Insect-Specific Virus Discovery: Significance for the Arbovirus Community. *Viruses*, 7(9): 4911–4928.
- Bouloy, M. and Weber, F. (2010). Molecular biology of rift valley Fever virus. *The Open Virology Journal*, 4: 8–14.
- Cavallari, M. L., Baltazar, F. N., Carvalho, E. C., Muñoz, D. R. and Tolezano, J. E. (2014). A Modified Shannon Trap for Use in Forensic Entomology. *Scientific Research Publishing Inc.*, (4): 69–75.
- CDC (2010). Anopheles Mosquitoes. Fact Sheet on Malaria. Available at: [<http://www.cdc.gov/malaria/about/biology/mosquitoes>] site visited on 22 December, 2015.

- CDC (2013). Viral Hemorrhagic Fevers (VHFs) *Bunyaviridae*. Available at [<http://www.cdc.gov/vhf/virus-families/bunyaviridae.html>] site visited on 2 August, 2015.
- Chengula, A. A., Mdegela, R. H. and Kasanga, C. J. (2013). Socio-economic impact of Rift Valley fever to pastoralists and agro pastoralists in Arusha, Manyara and Morogoro regions in Tanzania. *Springerplus*, 2: 549.
- Chipwaza, B., Mugasa, J. P., Selemani, M., Amuri, M., Mosha, F., Ngatunga, S. D. and Gwakisa, P. S. (2014). Dengue and Chikungunya Fever among Viral Diseases in Outpatient Febrile Children in Kilosa District Hospital, Tanzania. *PLoS Neglected Tropical Diseases*, 8(11).
- Coffey, L. L., Failloux, A. B. and Weaver, S. C. (2014). Review: Chikungunya virus-vector interactions. *Viruses*, 6(11): 4628–4663.
- Coffey, L. L., Forrester, N., Tsetsarkin, K., Vasilakis, N. and Weaver, C. S. (2013). Factors shaping the adaptive landscape for arbovirus: Implications for the Emergence of Disease. *National Institute of Health*, 8(2): 155–176.
- Cook, S., Diallo, M., Sall, A. A., Cooper, A. and Holmes, E. C. (2005). Mitochondrial markers for molecular identification of *Aedes* mosquitoes (Diptera: Culicidae) involved in transmission of arboviral disease in West Africa. *Journal of Medical Entomology*, 42: 19–28.

- Corby-Harris, V., Drexler, A., Watkins de Jong, L., Antonova, Y., Pakpour, N., Ziegler, R., Ramberg, F., Lewis, E. E., Brown, J. M., Luckhart, S. and Riehle, M. A. (2010). Activation of Akt signaling reduces the prevalence and intensity of malaria parasite infection and lifespan in *Anopheles stephensi* mosquitoes. *PLoS Pathogens*, (6).
- Corfas, A., Matthews, B. J., Ritchie, S. A., Vosshall, L. B. and Mcmeniman, C. J. (2013). Multimodal Integration of Carbon Dioxide and other sensory cues drives mosquito attraction to humans. *Cell*, 156(5): 1060–1071.
- Crochu, S., Cook, S., Attoui, H., Charrel, R. N., De Chesse, R., Belhouchet, M., de Micco, P. and de Lamballerie, X. (2004). Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of *Aedes spp.* mosquitoes. *Journal of General Virology*, 85: 1971–1980.
- Crump, J. A., Morrissey, A. B., Nicholson, W. L., Massung, R. F., Stoddard, R. A., Galloway, R. L. and Bartlett, J. A. (2013). Etiology of Severe Non-malaria Febrile Illness in Northern Tanzania: A Prospective Cohort Study. *PLoS Neglected Tropical Diseases*, 7(7).
- Daravath, S., Madhavi, Y. M., Chakrapani, P. and Reddy, N. B. (2013). Molecular Identification of *Aedes albopictus* (Diptera: Culicidae) and Quantitative Analysis of COI gene in South-Indian species. *International Journal of Current Microbiology and Applied Science*, 2(8): 102–109.

- Dash, A. P., Bhatia, R., Sunyoto, T. and Mourya, D. T. (2013). Emerging and re-emerging arboviral diseases in Southeast Asia. *Journal of Vector Borne Diseases*, 50(2): 77–84.
- DeFoliart, G. R. and Watts, D. M. (1987). Advances in Mosquito-Borne Arbovirus/Vector Research. *Annual Review of Entomology*, 32(1): 479.
- Drosten, C., Götting, S., Schilling, S., Panning, M., Schmitz, H., Asper, M. and Günther, S. (2002). Rapid Detection and Quantification of RNA of Ebola and Marburg Viruses, Lassa Virus, Crimean-Congo Hemorrhagic Fever Virus, Rift Valley Fever Virus, Dengue Virus, and Yellow Fever Virus by Real-Time Reverse Rapid Detection and Quantification of RNA. *Journal of Clinical Microbiology*, 40(7): 2323–2330.
- Englbrecht, C., Gordon, S., Venturelli, C. and Rose, A. (2015). Evaluation of BG-Sentinel Trap as a Management Tool to Reduce *Aedes albopictus* Nuisance in an Urban Environment in Italy. *Journal of the American Mosquito Control Association*, 31(1): 16–25.
- Eshoo, M. W., Whitehouse, C. A., Zoll, S. T., Massire, C., Pennella, T. T. D., Blyn, L. B. and Ecker, D. J. (2007). Direct broad-range detection of alphaviruses in mosquito extracts. *Virology*, 368(2): 286–295.
- Fagbo, S. F. (2002). The evolving transmission pattern of Rift Valley fever in the Arabian Peninsula. *Annals of the New York Academy of Sciences*, 969: 201–204.

- Gan, V. C. H. and Leo, Y. S. (2013). Current epidemiology and clinical practice in arboviral infections-implications on blood supply in the region. *ISBT Science Series*, 9: 262 - 267.
- Gao, X., Nasci, R. and Liang, G. (2010). The neglected arboviral infections in mainland China. *PLoS Neglected Tropical Diseases*, 4(4).
- Gautret, P., Simon, F., Hervius Askling, H., Bouchaud, O., Leparc-Goffart, I., Ninove, L. and Parola, P. (2010). Dengue type 3 virus infections in European travellers returning from the Comoros and Zanzibar, February-April 2010. *Euro Surveillance : Bulletin Européen Sur Les Maladies Transmissibles; European Communicable Disease Bulletin*, 15(15): 19541.
- Gorchakov, R., Frolova, E., Sawicki, S., Atasheva, S., Sawicki, D. and Frolov, I. (2008). A new role for ns polyprotein cleavage in Sindbis virus replication. *Journal of Virology*, 82(13): 6218–6231.
- Gratz, N. G. (2004). Critical review of *Aedes albopictus*. *Medical and Veterinary Entomology*, 18: 215–227.
- Gubler, D. and Trent, D. W. (1994). Emergence of epidemic dengue/dengue hemorrhagic fever as a public health problem in the Americas. *Infectious Agents Disease Journal*, (2): 383-93.
- Gubler, D. J. (2001). Human Arbovirus Infections Worldwide. *Annals of the New York Academy of Sciences*, 951(1): 13–24.

- Gubler, D. J. (2002). Epidemic dengue/dengue hemorrhagic fever as a public health, social and economic problem in the 21st century. *Trends in Microbiology*, 10(2): 100–103.
- Gubler, D. J. (2009). Vector-borne diseases, *Nature Review Microbiology*, 28(2): 583–588.
- Guzman, M. G., Halstead, S. B., Artsob, H., Buchy, P., Farrar, J., Gubler, D. J., Hunsperger, E., Kroeger, A., Margolis, H. S., Martínez, E., Nathan, M. B., Pelegrino, J. L., Simmons, C., Yoksan, S. and Peeling, R. W. (2010). Dengue : a continuing global threat. *Nature Review Microbiology*, 8(12): 7–16.
- Hall, R. A., Blitvich, B. J., Johansen, C. A. and Blacksell, S. D. (2012). Advances in arbovirus surveillance, detection and diagnosis. *Journal of Biomedicine and Biotechnology*, 12(5): 12–35.
- Harrington, L. C., Edman, J. D. and Scott, T. W. (2001). Why Do Female *Aedes aegypti* (Diptera: Culicidae) Feed Preferentially and Frequently on Human Blood. *Journal of Medical Entomology*, 38(3): 411–422.
- Heinrich, N. Elmar Saathoff, E., Nina Weller, N., Clowes, P., Kroidl, I., Ntinginya, E., Machibya, H., Maboko, L., Loscher, T., Dobler, G. and Hoelscher, M. (2012). High seroprevalence of Rift Valley fever and evidence for endemic circulation in Mbeya region, Tanzania, in a cross-sectional study. *PLoS Neglected Tropical Diseases*, 6(3): 1557.

- Hernandez, R., Brown, D. T. and Paredes, A. (2014). Structural differences observed in arboviruses of the *Alphavirus* and *Flavivirus* genera. *Advances in Virology*, (9): 1–24.
- Herrera, F., Urdaneta, L., Rivero, J., Zoghbi, N., Ruiz, J., Carrasquel, G., Martínez, J. A., Pernalete, M., Villegas, P., Montoya, A., Rubio-Palis, Y. and Rojas, E. (2006). Population genetic structure of the dengue mosquito *Aedes aegypti* in Venezuela. *Memorias do Instituto Oswaldo Cruz*, 101: 625–633.
- Himeidan, Y. E., Kweka, E. J., Mahgoub, M. M., El Rayah, E. A. and Ouma, J. O. (2014). Recent Outbreaks of Rift Valley Fever in East Africa and the Middle East. *Frontiers in Public Health*, 2(10): 1–11.
- Hoffmann, A. A., Montgomery, B. L., Iturbe-Ormaetxe, I., Johnson, P. H., Muzzi, F. and O'Neill, S. L. (2011). Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature*, 476(7361): 454–457.
- Hoffmann, P. R., Woodrow, R. J., Calimlim, P. S., Sciulli, R., Effler, P. V, Miyamoto, V., Imrie, A., Yanagihara, R. and Nerurkar, V. R. (2004) West Nile virus surveillance: A simple method for verifying the integrity of RNA in mosquito (Diptera: Culicidae) pools. *Journal of Medical Entomology*, 41: 731–5.
- Hopkins, G. H. E. (1952). *Mosquitoes of Ethiopian Region: Larval Bionomics of Mosquitoes and Taxonomy of Culicinae Larvae* (Vol. 2). Adlard and Son, Ltd, London, England.

- Horne, K. M. and Vanlandingham, D. L. (2014). Bunyavirus-vector interactions. *Viruses*, 6(11): 4373–4397.
- Isaacs, A. T., Jasinskiene, N., Tretiakov, M., Thiery, I., Zettor, A., Bourgouin, C. and James, A. A. (2012). Transgenic *Anopheles stephensi* coexpressing single-chain antibodies resist *Plasmodium falciparum* development. *Proceedings of the National Academy of Sciences, USA*, 109(19): 22–30.
- Ito, J., Ghosh, A., Moreira, L. A., Wimmer, E. A. and Jacobs-Lorena, M. (2002) Transgenic anopheline mosquitoes impaired in transmission of a malaria parasite. *Nature*, 4(17): 452–455.
- Johnson, N., Voller, K., Mansfield, K. and Fooks, A. R. (2012). Rapid Molecular Detection Methods for Arboviruses of Livestock of Importance to Northern Europe. *Journal of Biomedicine and Biotechnology*, 2(2): 1–5.
- Johnson, N., Wakeley, P. R., Mansfield, K. L., McCracken, F., Haxton, B., Phipps, L. P. and Fooks, A. R. (2010). Assessment of a novel real-time pan-flavivirus RT-polymerase chain reaction. *Vector Borne and Zoonotic Diseases*. Larchmont, New York, 10(7): 665–671.
- Juliano, S. A. (2009). Species Interactions Among Larval Mosquitoes: Context Dependence Across Habitat Gradients. *Annual Review of Entomology*, 54(1): 37–56.
- Jupp, P. G. and McIntosh, B. M. (1988). Chikungunya, a virus disease. In: *The Arboviruses*. (Edited by Monath, T. P.). Epidemiology and Ecology. American Public Health Association, Washington, D.C. pp33-36.

- Jupp, P. G., Grobbelaar, A. A., Leman, P. A., Kemp, A., Dunton, R. F., Burkot, T. R., Ksiazek, T. G. and Swanepoel, R. (2000). Experimental detection of Rift Valley fever virus by reverse transcription–polymerase chain reaction assay in large samples of mosquitoes. *Journal Medical Entomology*, 37: 467–71.
- Kamareddine, L. (2012). The biological control of the malaria vector. *Toxins*, 4(9): 748–767.
- Kean, J., Rainey, S., McFarlane, M., Donald, C., Schnettler, E., Kohl, A. and Pondeville, E. (2015). Fighting Arbovirus Transmission: Natural and Engineered Control of Vector Competence in *Aedes* Mosquitoes. *Insects*, 6(1): 236–278.
- Kifaro, E. G., Nkangaga, J., Joshua, G., Sallu, M., Dautu, G. and Kasanga, C. J. (2014). Epidemiological study of Rift Valley fever virus in Kigoma, Tanzania. *The Onderstepoort Journal of Veterinary Research*, 81(2): 1–5.
- Knowlton, N. and Weigt, L. A. (1998). New dates and new rates for divergence across the Isthmus of Panama. *Proceedings of the Royal Society B: Biological Sciences*, 265(1412): 2257.
- Krockel, U., Rose, A., Eiras, A. E. and Geier, M. (2006). New tools for surveillance of adult yellow fever mosquitoes: comparison of trap catches with human landing rates in an urban environment. *Journal of the American Mosquito Control Association*, 22(2): 229–238.

- Kumar, N. P., Rajavel, A. R., Natarajan, R. and Jambulingam, P. (2007). DNA barcodes can distinguish species of Indian mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology*, 44(1): 1–7.
- Kuno, G. and Chang, G. J. (2005). Biological Transmission of Arboviruses : Reexamination of and New Insights into Components, Mechanisms, and Unique Traits as Well as Their Evolutionary Trends. *Clinical Microbiological Reviews*, 18(4): 608–637.
- Kuno, G., Chang, G., Tsuchiya, K., Karabatsos, N. and Cropp, C. (1998). Phylogeny of the genus Flavivirus. *Journal of Virology*, 72(1): 73–83.
- Kuno, G., Mitchell, C. J., Chang, G. J. J. and Smith, G. C. (1996). Detecting bunyaviruses of the Bunyamwera and California serogroups by a PCR technique. *Journal of Clinical Microbiology*, 34(5): 1184–1188.
- Kweka, E. J., Owino, E. A, Mwang'onde, B. J., Mahande, A. M., Nyindo, M. and Mosha, F. (2011). The role of cow urine in the oviposition site preference of culicine and *Anopheles* mosquitoes. *Parasites and Vectors*, 4(1): 184.
- Kyle, J. L. and Harris, E. (2008). Global spread and persistence of dengue. *Annual Review of Microbiology*, 62: 71–92.
- LaBeaud, A. D. (2008). Why arboviruses can be neglected tropical diseases. *PLoS Neglected Tropical Diseases*, 2(6): 1–2.

- LaBeaud, A. D, Bashir, F. and King, C. (2011). Measuring the burden of arboviral diseases: the spectrum of morbidity and mortality from four prevalent infections. *Population Health Metrics*, 9(1): 1.
- Labuda, M. and Nuttall, P. (2004). Tick-borne viruses. *Parasitology*, 129: 221-245.
- Lakshmi, V., Neeraja, M., Subbalaxmi, M. V. S., Parida, M. M., Dash, P. K., Santhosh, S. R. and Rao, P. V. L. (2008). Clinical Features and Molecular Diagnosis of Chikungunya Fever from South India. *Clinical Infectious Diseases*, 46(9): 1436–1442.
- Lanciotti, R.S., Calisher, C. H., Gubler, D. J., Chang, G. J. and Vorndamt, A. V. (1992). Rapid Detection and Typing of Dengue Viruses from Clinical Samples by Using Reverse Transcriptase-Polymerase Chain Reaction. *Journal of Clinical Microbiology*, 30(3): 545–551.
- Leland, D. S. and Ginocchio, C. C. (2007). Role of cell culture for virus detection in the age of technology. *Clinical Microbiology Reviews*, 20(1): 49–78.
- Li, C. X., Smith, M. L., Fulcher, A., Kaufman, P. E., Zhao, T. Y. and Xue, R. D. (2015). Field Evaluation of Three New Mosquito Light Traps Against Two Standard Light Traps to Collect Mosquitoes (Diptera: Culicidae) and Non-Target Insects in Northeast Florida. *Florida Entomologist*, 98(1): 114–117.
- Liang, G., Gao, X. and Gould, E. A. (2015). Factors responsible for the emergence of arboviruses; strategies, challenges and limitations for their control. *Emerging Microbes and Infections*, 4(3): 18.

- Lindenbach, B. D. and Rice, C. M. (2003). Molecular Biology of *Flavivirus*. *Advances in Virus Research*, 59: 1317–1542.
- Linthicum, K. J., Davies, F. G., Bailey, C. L. and Kairo, A. (1984). Mosquito species encountered in a flooded grassland dambo in Kenya. *Mosquito News*, 44: 228–232.
- Lutomiah, J., Bast, J., Clark, J., Richardson, J., Yalwala, S., Oullo, D., Mutisya, J., Mulwa, F., Wurapa, E. and Sang, R. (2013). Abundance, diversity, and distribution of mosquito vectors in selected ecological regions of Kenya: Public health implications. *Journal of Vector Ecology*, 38(1): 134–142.
- Lwetoijera, D. W., Sumaye, R. D., Madumla, E. P., Kavishe, D. R., Mnyone, L. L., Russell, T. L. and Okumu, F. O. (2010). An extra-domiciliary method of delivering entomopathogenic fungus, *Metharizium anisopliae* IP 46 for controlling adult populations of the malaria vector, *Anopheles arabiensis*. *Parasites and Vectors*, 3(18): 5–10.
- Mansfield, K. L., Banyard, A. C., McElhinney, L., Johnson, N., Horton, D. L., Hernandez-Triana, L. M. and Fooks, A. R. (2015). Rift Valley fever virus: A review of diagnosis and vaccination, and implications for emergence in Europe. *Vaccine*, 33(42): 5520–5531.
- Marchette, N. J., Garcia, R. and Rudnick, A. (1969). Isolation of Zika virus from *Aedes aegypti* mosquitoes in Malaysia. *American Journal of Tropical Medicine and Hygiene*, 18: 411–415.

- Mboera, L. E. G., Mweya, C. N., Rumisha, S. F., Tungu, P. K., Stanley, G., Makange, M. R., Misinzo, G., De Nardo, P., Vairo, F. and Oriyo, N. M. (2016). The risk of dengue virus transmission in Dar es Salaam, Tanzania during an epidemic period of 2014. *PLoS Neglected Tropical Diseases*, 27: 1–15.
- Menzel, W., Jelkmann, W. and Maiss, E. (2002). Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. *Journal of Virological Methods*, 99: 81–92.
- Miller, N., Mettelman, R. C., Baker, S. C. and Harbison, J. E. (2015). Use of Centers for Disease Control and Prevention Gravid Trap in catch basins : Proof-of-Concept Trials. *Journal of the American Mosquito Control Association*, 31(2): 96–199.
- Mustafa, M. S., Rasotgi, V., Jain, S. and Gupta, V. (2014). Discovery of fifth serotype of dengue virus (DENV-5): A new public health dilemma in dengue control. *Medical Journal Armed forces India*, 7: 67 – 70.
- Mutebi, J., Crabtree, M. B., Kading, R. C., Powers, A. M. and Lutwama, J. J. (2012). Mosquitoes of Western Uganda. *Journal of Medical Entomology*, 49(6): 1289 - 1306.
- Mweya, C. N., Kimera, S. I., Mellau, L. S. B. and Mboera, L. E. G. (2015). Inter-epidemic abundance and distribution of potential mosquito vectors for Rift Valley fever virus in Ngorongoro district, Tanzania. *Global Health Action*, 8(1): 1–8.

- Nderitu, L., Lee, J. S., Omolo, J., Omulo, S., O'Guinn, M. L., Hightower, A. and Njenga, M. K. (2011). Sequential Rift Valley fever outbreaks in eastern Africa caused by multiple lineages of the virus. *The Journal of Infectious Diseases*, 203(5): 655–65.
- Ndiath, M. O., Mazenot, C., Gaye, A., Konate, L., Bouganali, C., Faye, O. and Trape, J. F. (2011). Methods to collect *Anopheles* mosquitoes and evaluate malaria transmission: a comparative study in two villages in Senegal. *Malaria Journal*, 10(1): 270.
- Ochieng, C., Lutomiah, J., Koka, H., Chepkorir, E., Yalwala, S. and Sang, R. (2013). Mosquito-borne arbovirus surveillance at selected sites in diverse ecological zones of Kenya; 2007 - 2012. *Virology Journal*, 10(1): 140.
- Parida, M., Posadas, G., Inoue, S., Hasebe, F. and Morita, K. (2004). Real-Time Reverse Transcription Loop-Mediated Isothermal Amplification for Rapid Detection of West Nile Virus Real-Time Reverse Transcription Loop-Mediated Isothermal Amplification for Rapid Detection of West Nile Virus. *Journal of Clinical Microbiology*, 257(1): 257–263.
- Pepin, M., Bouloy, M., Bird, B. H., Kemp, A. and Paweska, J. (2010). Rift Valley fever virus (Bunyaviridae: Phlebovirus): An update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. *Veterinary Research*, 41(6).
- Pialoux, G., Gaüzère, B.-A., Jauréguiberry, S. and Strobel, M. (2007). Chikungunya, an epidemic arbovirosis. *The Lancet Infectious Diseases*, 7(5): 319–327.

- Powers, A. M. and Roehrig, J. T. (2011). Diagnostic Virology Protocols. In R. J. Stephenson and A. Warnes (Eds.). *Humana Press*, Totowa, NJ. pp 17–38.
- Ratcliffe, W. M. (2004). Microbe-vector Interactions in Vector-borne Diseases. In S. H. Gillespie (Ed.), *Cambridge University Press, Cambridge*. pp. 199–262.
- Raval-Nelson, P., Soin, K. and Tolerud, S. (2005). Analysis of *Bacillus sphaericus* in controlling mosquito populations in urban catch basins. *Journal of Environmental Health*, 67(7): 28–31.
- Reisen, W. K., Meyer, R. P., Tempelis, C. H. and Spoehel, J. J. (1990). Mosquito abundance and bionomics in residential communities in Orange and Los Angeles Counties, California. *Journal of Medical Entomology*, 27: 356-367.
- Roiz, D., Vázquez, A., Seco, M. P. S., Tenorio, A. and Rizzoli, A. (2009). Detection of novel insect flavivirus sequences integrated in *Aedes albopictus* (Diptera: Culicidae) in Northern Italy, *Virology Journal* 6(93).
- Romoser, W., Oviedo, M., Lerdthusnee, K., Patrican, L., Turell, M., Dohm, D. and Bailey, C. (2011). Rift Valley fever virus-infected mosquito ova and associated pathology: possible implications for endemic maintenance. *Research and Reports in Tropical Medicine*, 2: 121–127.
- Rueda, L. M. (2004). Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with dengue virus transmission. *Zootaxa; Magnolia Press. Auckland, NZ*, (589): 1–6.

- Rueda, L. M. (2008). Global diversity of mosquitoes (Insecta: Diptera: Culicidae) in freshwater. *Hydrobiologia*, 595(1): 477–487.
- Samuel, P. P. and Tyagi, B. K. (2006). Diagnostic methods for detection and isolation of dengue viruses from vector mosquitoes. *Indian Journal of Medical Research*, 123(5): 615–628.
- Sanger, F., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America*, 74(12): 5463–5467.
- Schmaljohn, A. L. and McClain, D. (1996). *Alphaviruses* (Togaviridae) and Flaviviruses (Flaviviridae). In B. S (Ed.), *Medical Microbiology* (4th ed., p. Chpt 54). Galveston (TX): University of Texas. Galveston.
- Scholte, E. J., Ng'habi, K., Kihonda, J., Takken, W., Paaijmans, K. and Knols, B. G. J. (2005). An entomopathogenic fungus for control of adult African malaria mosquitoes. *Science*, 308(5728): 1641–1642.
- Shu, P. and Huang, J. (2004). Current Advances in Dengue. *Clinical and Diagnostic Laboratory Immunology*, 11(4): 642–650.
- SIB (Swiss Institute of Bioinformatics) (2015). *Bunyaviridae* Genome. [http://viralzone.ex-pasy.org/all_by_species/82.html] site visited on 26/09/2015.
- Sindato, C., Karimuribo, E. D., Pfeiffer, D. U., Mboera, L. E. G., Kivaria, F., Dautu, G., Bernard, B. and Paweska, J. T. (2014). Spatial and temporal pattern of rift valley fever outbreaks in Tanzania; 1930 to 2007. *PLoS ONE*, 9(2).

- Sikka, V., Chattu, V. K., Popli, R. K., Galwankar, S. C., Kelkar, D., Sawicki, S. G. and Papadimos, T. J. (2016). Review: The Emergence of Zika Virus as a Global Health Security Threat. *Journal of Global Infectious Diseases*, 8(1): 3–15.
- Simpson, E. H. (1949). Measurement of diversity. *Nature*, 163: 688.
- Smallegange, R. C., Verhulst, N. O. and Takken, W. (2011). Sweaty skin: An invitation to bite? *Trends in Parasitology*, 27(4): 143–148.
- Smith, D. R., Lee, J. S., Jahrling, J., Kulesh, D. A., Turell, M. J., Groebner, J. L. and O’Guinn, M. L. (2009). Development of field-based real-time reverse transcription-polymerase chain reaction assays for detection of Chikungunya and O’nyong-nyong viruses in mosquitoes. *American Journal of Tropical Medicine and Hygiene*, 81(4): 679–684.
- Smith, K. M., Nanda, K., Spears, C. J., Ribeiro, M., Vancini, R., Piper, A. and Hernandez, R. (2011). Structural mutants of dengue virus 2 transmembrane domains exhibit host-range phenotype. *Virology Journal*, 8(1): 289.
- Soliman, H. and El-Matbouli, M. (2006). Reverse transcription loop-mediated isothermal amplification for rapid detection of viral hemorrhagic septicaemia virus (VHS). *Veterinary Microbiology*, 114(3-4): 205–213.
- Sow, A., Loucoubar, C., Diallo, D., Faye, O., Ndiaye, Y., Senghor, C. S. and Sall, A. A. (2016). Concurrent malaria and arbovirus infections in Kedougou, southeastern Senegal. *Malaria Journal*, 15(1): 47.

- Strauss, J. H. and Strauss, E. G. (1994). The alphaviruses: gene expression, replication, and evolution. *Microbiological Reviews*, 58(3): 491–562.
- Sumaye, R. D., Abatih, E. N., Thiry, E., Amuri, M., Berkvens, D. and Geubbels, E. (2015). Inter-epidemic Acquisition of Rift Valley Fever Virus in Humans in Tanzania. *PLoS Neglected Tropical Diseases*, 9(2).
- Thenmozhi, V., Mariappan, T., Krishnamoorthy, R., Baskarn, G., Krishnamoorthi, R., Balaji, T. and Tyagi, B. K. (2014). A first note on Japanese encephalitis virus isolation from *Culex quinquefasciatus* in Northern West Bengal. *International Journal of Mosquito Research*, 1(1): 1–4.
- Thier, A. (2001). Balancing the risks: Vector control and pesticide use in response to emerging illness. *Journal of Urban Health*, 78(2): 372–378.
- URT (2013). 2012 Population and Housing Census Population, the Republic of Tanzania. [<http://www.google.co.tz>] site visited on 02/03/16.
- Vector - Illustration showing the life cycle of a mosquito. [www.123rf.com/photo] site visited on 26 October, 2015.
- Walton, C. (1999). Molecular identification of mosquito species. *Biological Journal of the Linnean Society*, 68(1-2): 241–256.
- Walton, C., Handley, J. M., Kuvangkadilok, C., Collins, F. H., Harbach, R. E., Baimai, V. and Butlin, R. K. (1999). Identification of five species of the *Anopheles dirus* complex from Thailand, using allele-specific polymerase chain reaction. *Medical and Veterinary Entomology*, 13(1): 24–32.

- Weaver, S. C. and Reisen, W. K. (2010). Present and future arboviral threats. *Antiviral Research*, 85(2): 328–345.
- Weissenböck, H., Hubalek, Z., Bakonyi, T. and Nowotny, N. (2010). Zoonotic mosquito-borne flaviviruses: Worldwide presence of agents with proven pathogenicity and potential candidates of future emerging diseases. *Veterinary Microbiology*, 140(4): 271–280.
- Wensman, J. J., Lindahl, J., Wachtmeister, N., Torsson, E., Gwakisa, P., Kasanga, C. and Misinzo, G. (2015). A study of Rift Valley fever virus in Morogoro and Arusha regions of Tanzania - serology and farmers' perceptions. *Infection Ecology and Epidemiology*, 5(6): 0025.
- Whitehead, S. S., Blaney, J. E., Durbin, A. P. and Murphy, B. R. (2007). Prospects for a dengue virus vaccine. *Nature Review Microbiology*, 5(7): 518–528.
- WHO (2004). Global Strategic Framework For Integrated Vector Management. World Health Organization, Geneva: [http://whqlibdoc.who.int/hq/2004/WHO_CDS_CPE_PVC_2004_10.pdf] site visited on 20 October 2015.
- WHO (2014). Dengue outbreak in the United Republic of Tanzania (Situation as of 30 May 2014). [<http://www.afro.who.int/>] site visited on 25 October 2015
- WHO (2016). Zika virus technical report. Interim Risk Assessment, WHO European [www.euro.who.int/] site visited on 25 July, 2015.

Wilkerson, R. C., Linton, Y. M., Fonseca, D. M., Schultz, T. R., Price, D. C. and Strickman, D. A. (2015). Making Mosquito Taxonomy Useful: A Stable Classification of Tribe *Aedini* that Balances Utility with Current Knowledge of Evolutionary Relationships, *Plos ONE*, 10(7).

Xiao, G., Ying, S. H., Zheng, P., Wang, Z. L., Zhang, S., Xie, X. Q. and Feng, M. G. (2012). Genomic perspectives on the evolution of fungal entomo-pathogenicity in *Beauveria bassiana*. *Scientific Reports*, (2): 483.

Zhdanov, V. (1975). Integration of viral genomes. *Nature*, 256: 471–473.

APPENDICES

Appendix 1: Extraction of Viral RNA (Modified Qiagen Protocol)

Procedure

1. Place the base of the 1.5 mL Eppendorf tube containing mosquitoes in liquid nitrogen until sizzling stop. Grind briefly using a plastic application stick and then add 250 μ L of PBS and mix gently.
2. Add 140 μ L of the supernatant of the pulverized mosquito (remember to cut the tip of the pipette tip) to 560 μ L of prepared Buffer AVL in the 1.5 mL microcentrifuge tube and pulse vortex.
3. Digest the suspension with 0.5 mg 20 μ L proteinase K and incubate at room temperature (15 – 25°C) for 10 min and briefly centrifuge (to remove drops from the inside of the lid).
4. Add 560 μ L of ethanol (96–100%) to the sample, and mix by pulse-vortexing and briefly centrifuge (to remove drops from inside the lid).
5. Place the QIAamp Mini column into a clean 2 mL collection tube and carefully apply 630 μ L of the solution from step four to the QIAamp Mini column without wetting the rim. Centrifuge at 6 000 x g (8 000 rpm) for one min and discard the tube containing the filtrate. Repeat step five if there are some sample left in the tube.
6. Place the QIAamp Mini column in a clean 2 mL collection tube, and add 500 μ L of Buffer AW1 to the QIAamp Mini column. Centrifuge at 6 000 x g (8 000 rpm) for one min. and discard the tube containing the filtrate.
7. Add 500 μ L of Buffer AW2 to the QIAamp Mini column and centrifuge at full speed (20 000 x g; 14 000 rpm) for three min.

8. Placed the QIAamp Mini column in a clean 1.5 mL microcentrifuge tube and add 60 μ L of Buffer AVE to the QIAamp Mini column and incubate at room temperature for 1 min. Centrifuge at 6 000 x *g* (8 000 rpm) for one min.
9. Store the RNA extracted in the -20 freezer

Appendix 2: QIAquick Gel Extraction Kit

Procedure:

1. The gel was placed on the UV illuminator and the DNA fragment excised from the agarose gel with a clean sharp scalpel.
2. The gel slices weighed into an eppendorf tube and three volumes of Buffer QG was added to 0 volume of gel.
3. Incubated at 50°C vortex the tube every 2–3 min until the gel slice has completely dissolved
4. Add 1 gel volume isopropanol to the sample and mix.
5. Place a QIAquick spin column in a provided 2 mL collection tube. Apply the sample to the QIAquick column and centrifuge for 1 min.
6. Add 500 μ L Buffer QG to the QIAquick column and centrifuge for 1 min. Discard flow-through and place the QIAquick column back into the same tube.
7. To wash, add 750 μ L Buffer PE to QIAquick column and allow to stand for 5 min and centrifuge for 1 min. Discard flow-through.
8. Place QIAquick column into a clean 1.5 mL microcentrifuge tube.
9. To elute DNA, add 30 μ L Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the QIAquick membrane, let the column stand for 4 min and centrifuge the column for 1 min.
10. Store the eluted DNA in -20 or can be used immediately.

Appendix 3: GenElute™ PCR Clean-Up Kit - (Sigma-Aldrich)**Procedure**

1. Insert a GenElute plasmid mini spin column into a provided collection tube. Add 500 μ L of the Column Preparation Solution to each mini spin column and centrifuge at 12 000 x g for 1 minute. Discard the eluate.
2. Add 5 volumes of Binding Solution to 1 volume of the PCR reaction and mix. Transfer the solution into the binding column. Centrifuge the column at maximum speed (16 000 Xg) for 1 minute. Discard the eluate, but retain the collection tube.
3. Replace the binding column into the collection tube. Apply 500 μ L of diluted Wash Solution to the column and centrifuge at maximum speed for 1 minute. Discard the eluate, but retain the collection tube.
4. Replace the column into the collection tube. Centrifuge the column at maximum speed for 2 minutes, without any additional wash solution, to remove excess ethanol. Discard any residual eluate as well as the collection tube.
5. Transfer the column to a fresh 2 mL collection tube. Apply 50 μ L of Elution Solution or water to the centre of each column. Incubate at room temperature for 1 minute.
6. To elute the DNA, centrifuge the column at maximum speed for 5 minute and use either immediately or store at -20 °C.

Appendix 4: Mosquito Collection Form

Collection No:	Latitude/Longitude	Date:	Time:
District/Division	Village/Urban	Specific Location	
Elevation	Name of Collector		
Topography <input type="radio"/> Mountain <input type="radio"/> Hilly <input type="radio"/> Plain <input type="radio"/> Valley	Hosts <input type="radio"/> Human <input type="radio"/> Livestock Sky <input type="radio"/> Clear <input type="radio"/> Partly cloudy <input type="radio"/> Fog	Larval Habitat <input type="checkbox"/> Pond <input type="checkbox"/> Ground pool <input type="checkbox"/> Marsh <input type="checkbox"/> Ditch <input type="checkbox"/> Tree hole <input type="checkbox"/> Rice field <input type="checkbox"/> Well <input type="checkbox"/> Ditch <input type="checkbox"/> Rock pool Other: _____	
Environment <input type="radio"/> Forest <input type="radio"/> Swamp <input type="radio"/> Mangrove <input type="radio"/> Bush <input type="radio"/> Urban <input type="radio"/> Village	Wind <input type="radio"/> None <input type="radio"/> Light <input type="radio"/> Gusts <input type="radio"/> Strong	Water/water movement <input type="checkbox"/> Permanent <input type="checkbox"/> Temporal <input type="checkbox"/> Standing <input type="checkbox"/> Flowing	
Collection Method <input type="radio"/> Resting - House - Animal Shelter - Tree hole - Vegetation <input type="radio"/> Landing - Human/Animal <input type="radio"/> Traps - Light - BG - Hoovers Other: _____	Distance from Homes _____m Environmental Modifiers <input type="radio"/> Agriculture <input type="radio"/> Pasture <input type="radio"/> Plantation <input type="radio"/> Other: _____	Turbidity <input type="checkbox"/> Clear <input type="checkbox"/> Turbid <input type="checkbox"/> Polluted Aquatic Vegetation <input type="checkbox"/> Submerged <input type="checkbox"/> Floating <input type="checkbox"/> Emergent <input type="checkbox"/> Submerged and Floating <input type="checkbox"/> Submerged and Emergent <input type="checkbox"/> Floating and Emergent	
Any other Ecological features			
Animals			