MOSQUITO DIVERSITY AND VIRUS INFECTION IN KILOMBERO VALLEY IN SOUTH-EASTERN TANZANIA

MARIANA JOHN SHAYO

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

2015

ABSTRACT

Mosquito-borne viruses are an important cause of human and domestic diseases worldwide. As crucially important emerging pathogens, they have caused multiple, notable and unnoticed epidemics of human disease over recent decades. All mosquito-borne viruses circulate within sylvatic cycles among wildlife and forest mosquitoes and may cause diseases in rural and urban populations after spillover transmission to human and domestic animals. The aim of this study was to determine mosquito diversity and transmission of Rift Valley fever virus (RVFV), dengue virus (DENV) and Chikungunya virus (CHIKV) in South-eastern Tanzania. In the present cross-sectional study, a total of 1320 adult mosquitoes were collected using Biogent (BG) sentinel traps and battery-powered aspirators. In addition, a total of 31 larvae were collected using a dipping technique and allowed to hatch into adults. Morphological identification of mosquitoes showed the presence of 12 different species which were unevenly distributed among different ecological zones within the two districts. Culex mosquitoes were found to be dominant at all mosquito collection sites and represented 89.1% followed by 10.1% Aedes and 0.8% Mansonia. Aedes mosquitoes were pooled into groups of 20 mosquitoes resulting into a total of 12 pools. No mosquito pool was positive for DENV or CHIKV when Aedes mosquito pools were screened for these viruses. However, RVFV was found in two pools of mosquitoes including one of *Aedes aegypti* and another of *Aedes pembaensis*. Furthermore, when Aedes mosquito pools were screened, eight pools were positive for Flavivirus and six pools were positive for Alphavirus respectively. The positivity to Flavivirus and Alphavirus with concurrent absence of DENV and CHIKV in these mosquitoes indicates the presence of viruses other than DENV and CHIKV. Results obtained from this study show that mosquitoes in Kilombero Valley carry viruses of

serious public health importance. It is recommended that mosquito-borne viruses be screened in febrile patients presenting at points of care in order to improve prognosis.

DECLARATION

I, Mariana John Shayo, 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.

_____ _____

Mariana Shayo Date

(MSc. Candidate)

The declaration is hereby confirmed;

_____ _____

Prof. Gerald Misinzo Date

(Supervisor)

COPYRIGHT

ACKNOWLEDGEMENTS

Special thanks to the Almighty God, who gives skills, knowledge and wisdom, and who has brought me this far. I wish to express my heartfelt gratitude to my supervisor, Prof. Gerald Misinzo of Department of Veterinary Microbiology and Parasitology at Sokoine University of Agriculture, Morogoro, Tanzania and Dr. Pertus Jansen Van Vuren of National Institute for Communicable Diseases in Special Pathogen Unit (NICD-SPU) (Republic of South Africa) for their priceless guidance and support throughout the course of this research.

My appreciation goes to all staff at National Institute for Communicable Diseases in Special Pathogen Unit (NICD-SPU) (Republic of South Africa), Prof. Janusz T. Paweska, Dr. Pertus Jansen Van Vuren, Dr. Jacqueline Weyer, Mr. Alen Kamp and Dr. Antoinette A. Grobbelaar for their scientific support in this research while I was at NICD-SPU Johannesburg, South Africa as well as at SUA, Tanzania they worked hard to ensure that I succeeded.

I also wish to express my sincere gratitude to Wellcome Trust through Southern African Centre for Infectious Disease Surveillance (SACIDS) for their financial support of my postgraduate studies.

DEDICATION

This work is dedicated to Almighty God. To my parents John and Geneveive Shayo for their distinguished and unwavering support. This was a great motivation for me to complete this work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

◦C	degree Celsius
µl	microliter
Ae.	Aedes
An.	Anopheles
bp	base pair
cDNA	complementary deoxyribonucleic acid
CHIKV	Chikungunya virus
Cx.	Culex
DENV	dengue virus
DNA	deoxyribonucleic acid
EEE	Eastern equine encephalomyelitis
LAC	La Crosse encephalitis
Ma.	Mansonia
MRCC	Medical Research Coordinating Committee
NICD	National Institute for Communicable Diseases
NSP	non-structural protein
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
SACIDS	Southern African Centre for Infectious Disease Surveillance
SLE	St. Louis encephalomyelitis

SPU	Special Pathogen Unit
SUA	Sokoine University of Agriculture
TAE	Tris Acetic ethylenediamine tetracetic acid buffer
WEE	Western equine encephalomyelitis
WNFV	West Nile fever virus
YFV	Yellow fever virus

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Febrile illnesses remain to be one of the common clinical presentations among person seeking healthcare in many developing countries despite a decline in malaria (Feikin *et al.,* 2011). Malaria has remained the most reason of fever and high mortalities in Tanzanian population for many years (Mboera *et al.,* 2013). However, despite successful malaria control and cutback in its transmission, prevalence and mortality in recent years, the number of patients presenting with fever have remained at the same level (Chipwaza *et al.,* 2014b). Most fevers are misdiagnosed as malaria leading to overuse of antimalarial drugs and poor prognosis among febrile patients (Russell *et al.,* 2010). Diverse aetiologies ranging from bacteria, viruses, parasites and fungi cause fever, making it difficult to diagnose febrile illnesses based on their clinical presentations (Chipwaza *et al.,* 2014b). Due to limited diagnostic tools at the point of care in developing countries, determination of actual causes of fevers based on clinical presentation has led to underreporting of most causes of febrile illnesses other than malaria (Chipwaza *et al.,* 2014a).

Most of the arthropod-borne viruses (arboviruses) cause febrile illnesses. Arboviruses are transmitted among vertebrates host through blood feeding by arthropod vectors like mosquitoes, flies and ticks (Weaver and Reisen, 2010). Arboviruses include only one taxa of DNA virus, the Asfarvirus that cause hemorrhagic febrile illness in pigs while the other arboviruses are RNA viruses belonging to Alphaviruses, Flaviviruses, Bunyaviruses, Nairoviruses, Phleboviruses, Orbiviruses, Vesiculoviruses and Thogotoviruses (Weaver and Reisen, 2010). Mosquitoes are the most important vectors in the spread of arboviral

disease in wild and domestic animals as well as in humans (Gubler, 2001). Viruses transmitted through mosquito bite are called mosquito-borne viruses. The adaptation of mosquitoes to spread various diseases depends on various factors such as feeding habit of mosquitoes, mosquito prevalence, density and distribution. About 3500 different mosquito species have been recorded worldwide (Harbach, 2015).The present study determines the mosquito diversity and transmission of RVFV, CHIKV and DENV in mosquitoes of Kilombero Valley South-eastern Tanzania.

1.2 Problem Statement and Justification

Kilombero and Ulanga districts are located within Morogoro region in South-Western Tanzania. Kilombero district has a population of 407 880 while Ulanga district has a population of 265 203 according to national census of 2012 (URT, 2014). Kilombero and Ulanga districts are located within the Kilombero river valley which is seasonally flooded up to 52 km wide at high waters (Sumaye *et al*., 2013; Charlwood *et al.,* 2000). On North-western side of the valley, there is a steep escarpment of Udzungwa mountains and grass-covered Mahenge mountains while Selous game reserve is on South-Eastern side. The Kilombero valley has a diverse ecology and demography with villages extending to the margins of flood plain where rice cultivation is the principal economic activity (Sumaye *et al*., 2013). The valley provides breeding sites for mosquitoes and an interface for interaction between human, domestic animals and wildlife. Lwetoijera *et al*. (2014) reported the intense malaria transmission in Kilombero river valley. In addition, intra-epidemic current transmission of RVFV in animals has been also reported in Kilombero river valley (Sumaye *et al.,* 2013). No report has been provided to explain the presence of Mosquito-borne viruses such as DENV and CHIKV in Kilombero and Ulanga districts.

1.3 Objectives

1.3.1 Overall objective

To determine mosquito diversity and transmission of mosquito-borne viruses in Kilombero Valley in Tanzania.

1.3.2 Specific objectives

i. To determine mosquito abundance in Kilombero Valley and

ii. To determine the infection rate of RVFV, CHIKV and DENV viruses in the mosquitoes.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mosquitoes

Mosquito are classified into order Diptera, sub-order Nematocera in Culicidae family. The family Culicidae is divided into three subfamilies including; Anophelinae, Culicinae and Toxorhynchitinae. Adult females of Anophelinae and that of Culicinae are blood feeders while those of Toxorhynchitinae feed on nectar and other plant juices (Harbach, 2007). Toxorhynchitinae are considered to have less importance in disease transmission in contrast to Anophelinae and Culicinae mosquitoes due to their feeding habit (Harbach, 2007). The Anophelinae subfamily comprise of three genera while Culicinae has 41 genera (Harbach, 2007).

2.1.1 Life cycle of mosquitoes

2.1.2 Mosquito diversity in Tanzania

Mosquito diversity in Tanzania remains unclear up to date, however a numbers of research have been conducted and some of mosquito species have been identified, such species are *Anopheles gambiae, An. pharoensis, An. coustani, An. maculipalpis, An. marshallii, Culex quinquefasciatus, Cx. unnivittatus, Mansonia uniformis* and *M. africana* (Mwanziva *et al.,* 2011). Lwetoijera *et al.* (2014) reported presence of *An. funestus, An. rivorulum*, *An. leesoni* and *An. arabience* in Kilombero Valley. *Aedes aegypti* in Kilombero Valley have been reported by Mweya *et al.* (2015). Other species identified in Ngorongoro include, *Culex pipiens complex, Cx. antennatus, Cx. tigripes, Cx. annulioris, Cx. cinereus, An. arabiensis and An. squamosus (*Mhina *et al.,* 2015).

2.1.3 Mosquito identification techniques

Mosquito species can be identified through different ways including morphological identification and DNA barcoding method. Morphological identification using

identification key is most used method where mosquitoes are identified based on observed feature on their body parts (Gillies and De Meillon, 1968). Several dichotomous key of all stages of mosquitoes have been developed for mosquito identification. However, keys for mosquito identification have also been developed (Gillies and De Meillon, 1968; Hopkins, 1952). DNA barcoding is a recent developed mosquito identification technique. The technique involves use of mosquito part as source of genetic information. Technique allows life's discrimination through analysis of small genome segment by exploiting diversity among DNA sequences which are viewed as genetic 'barcodes' that are fixed in species (Allander *et al.,* 2001). The technique is also used for identification of degraded mosquito (Krzywinski and Besansky, 2003).

2.1.4 Mosquito control

The use of insecticide treated nets (ITNs) has been the main mosquito control method in Tanzania. In recent days, there has been an increase in the household ownership of insecticide treated mosquito nets which is highly attributed to government initiatives and donor programmes to distribute nets free of charge to households or to vulnerable groups (Renggli *et al.,* 2013). The use of indoor residual spraying (IRS) in Tanzania has been adapted to complement ITNs in epidemic-prone districts. IRS was initially limited to epidemic-prone areas of the Kagera region in north-western Tanzania (Mashauri *et al.,* 2013). Other mosquito control measures including screening of houses have received little attention. A larger proportion of the population in rural area live in poorly constructed houses and only a few houses have windows with mosquito gauze. Kirby *et al*. (2009) reported the significantly effects of house design on incidence of malaria infection.

Mosquito control through larval source management (LSM) is limited some areas. So far, the use of larviciding has been restricted to Dar es Salaam. However, with current

technology, biological controls of larvae mosquito which involve *Bacillus thuringiensis* (Bt) have been developed (Mboera *et al.,* 2014). Mosquito control methods by the elimination of mosquito breeding sites through interfering with standing water is the most important methods to reduce mosquito abundance in an area. However, individuals need protect themselves from mosquito bite through wearing of long-sleeved shirts and full-length trousers and use of mosquito repellant.

2.2 Mosquito-borne viruses

Mosquito-borne viruses are biologically or mechanically transmitted from to mosquitoes and animals/human. Biological transmission requires virus replication in its vector prior to transmission to another susceptible host (Kuno and Chang, 2005). Mosquito-borne viruses are either transmitted vertically, involving passage of virus from infected mosquito to male and female offspring or horizontal transfer which involves the transmission between infected male and other female however, horizontal transmission is less common (Kuno and Chang, 2005). Mosquito-borne virus and other arboviruses circulate among wild animals and cause disease spillover transmission to domestic animal/human which are dead-end host (Gubler, 2001). Virus such as CHIKV and DENV are adapted to public amplification due to their corresponding vector adaptation to artificial breading sites, hence they now produce extensive epidemics (Weaver and Reisen, 2010). Mosquito-borne viruses include, CHIKV, DENV, RVFV, West Nile virus (WNFV), Yellow fever virus (YFV), St. Louis encephalitis (SLE), eastern equine encephalomyelitis (EEE), western equine encephalomyelitis (WEE), La Crosse encephalitis (LAC) and O'nyong'nyong virus (ONN) (Weaver and Reisen, 2010).

2.2.1 Virus distribution and replication in mosquito upon infection

Naturally, transmission of mosquito-borne viruses is maintained by ability of viruses to infect mosquitoes efficiently with the spread to, and replication in salivary gland of

mosquito (Gubler, 2001). However, not all mosquitoes get infected after getting infectious blood meal, nevertheless, not all infected mosquitoes can transmit the virus. For a mosquito to be able to transmit disease, the virus must replicate in salivary glands and disseminate beyond midgut. Incubation period between taking viremic blood meal and ability to transmit infection depends on rapid virus replication and dissemination in mosquito salivary gland (Gubler, 2001). Susceptibility of mosquito to virus infection varies widely with different geographical location and even within individuals of same strain (Gubler and Rosen, 1976).

2.2.2 Rift Valley fever

2.2.2.1 Rift Valley fever virus

Rift Valley fever virus is member of Phlebovirus genus in Bunyaviridae family. The virus is tri-segmented, single stranded negative sense RNA genome, with large (L), medium (M) and small (S) segments (Fig. 1). Segment L encodes the viral RNA-RNA dependent RNA polymerase. M segment encodes four proteins including two envelope glycoproteins (G1 and G2) and nonstructural proteins 14 K and 78K. The S segment encodes for a nucleocapsid (N) protein and other nonstructural proteins (Elliott *et al*., 1991). RVFV has genomic rearrangement via RNA segment re-assortment and homologous recombination which are both potential in promotion of genetic diversity. Generally, there are seven genetic lineages of RVFV, having no correlation with geographical locations (Bird *et al.,* 2007). Bird *et al.* (2007) reported re-assortment of RNA genome segment among viruses of Bunyaviridae family and re-assortment among RVFV strains.

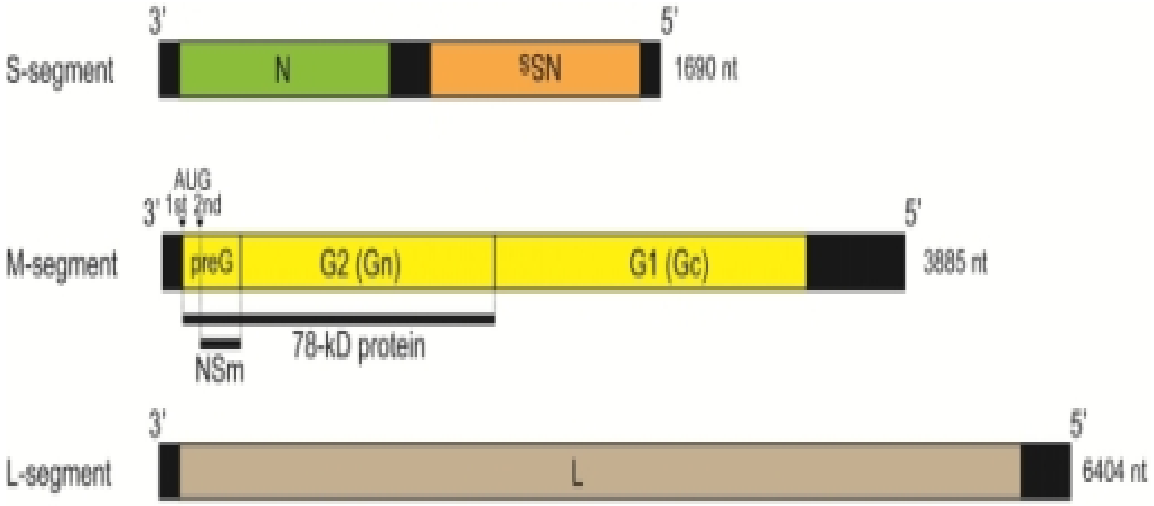

Figure 1: The genomic organization of RVFV genome. Source: Ikegami and Makino, (2011).

2.2.2.2 Rift Valley fever

Rift Valley fever outbreaks have been known to occur in duration of 5 to 20 years following heavy rainfall in Eastern and Southern Africa (Ibrahim *et al.,* 2008). Different mosquito species are involved in transmission of RVFV including, *Ae. aegypti, Ae. ochraceus, Ae. vexans, Ae. simpsoni, Ae. pembaensis, Cx. poicilipes, Cx. quinquefasciatus, Cx.univittatus, Cx. bitaeniorhynchus, An. squamosus, M. uniformis; and M. africanus* in East Africa (Sang *et al.,* 2010). *Ae. juppi, Ae. caballus, Ae. linneatopennis, Cx. pipiens, Ae. vexans, Ae. ochraceus and Ae. dalzieli* in West Africa (Fontenille *et al.,* 1998).

In domestic animals, RVFV is transmitted either through bite with infected mosquitoes or direct contact with infected animal tissues, body fluids and fomites. In human, various transmission routes have been reported. However, they vary in their contribution based on epidemic stages. Bite with RVF infected mosquito is predominant at first stages of outbreak while direct contact with infected animal is predominant during disease amplification stage (Pe'pin *et al.,* 2010). Other transmission routes include, direct contact with infected animal tissues, blood, or other body fluids and aerosols (Woods *et al.,* 2002).

RVFV persists in an environment through vertical transmission within mosquitoes and horizontal transmission between mosquitoes and animals. Principal reservoir of RVFV is not yet known but rodents are suspected hosts (Olive *et al.,* 2012). The disease is associated with massive abortion in animals while in human the disease is more often asymptomatic or with self-limiting, influenza-like illness characterized by fever, headache, muscular pain, vomiting and extreme weight loss. However, some patient experience complications which manifest as hemorrhages, retinitis and encephalitis (Mohamed *et al.,* 2010).

Rift Valley fever is becoming a global problem and especially in East Africa where several outbreaks have been reported. In Tanzania, several epidemics have been reported, that occurred in Ngorongoro in 1997-1998 and more recently 2006-2007 that was reported in 25 district of Arusha, Manyara, Kilimanjaro, Tanga, Dodoma, Iringa and Morogoro (Chengula *et al.,*2013; Sindato *et al*., 2011;).

2.2.3 Dengue

2.2.3.1 Dengue virus

Dengue virus is classified into Flavivirus in the Flavividae family (Thiel *et al.,* 2005). The virus has icosahedral envelope organization and spherical nucleocapsid core. Viral genome is positive sense-single stranded RNA with 11kb long, encode one open reading frame which is then translated into three different structural proteins; capsid (C), Membrane/ premembrane (M/PrM) and envelope (E) proteins and seven non structure proteins; NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5(Fig. 2) (Gebhard *et al.,* 2011).

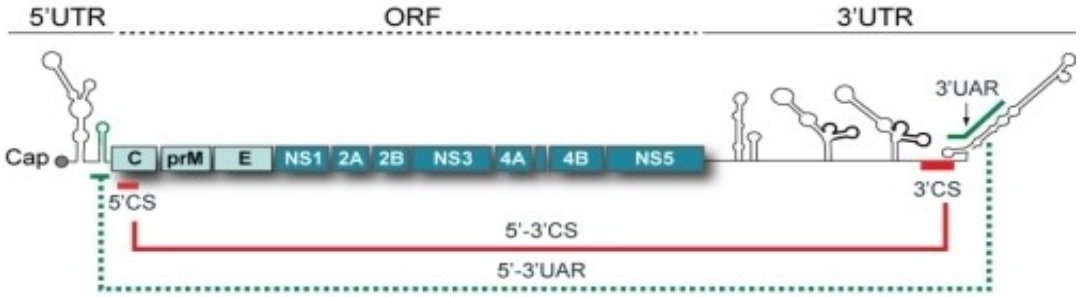

Figure 2: Schematic representation of the DENV genome. Source: Gebhard *et al.* (2011).

2.2.3.2 Dengue disease

Two fifths of world populations are at risk for dengue virus infection. About 50 million new cases are reported to occur worldwide every year (WHO, 2002).DENV infection is underreported in Tanzania. Moi *et al.* (2010) reported the isolation of DENV 3 from two travelers from Tanzania. Recently, massive dengue outbreak was reported in United Republic of Tanzania where 1017 individuals were infected (WHO, 2014). There are four serologically distinct but closely related DENV (serotype 1-4). Infection with any DENV serotype may result into asymptomatic or dengue fever and dengue hemorrhagic fever/dengue shock syndrome. In addition, infection with one DENV serotype provide an individual with long time protection of same virus but short time protection to other virus serotype (Gibbons and Vaughn, 2002). However, sequential infection may increase risk for sever disease (Nimmannitya, 1993). Young children upon DENV infection they develop differentiation febrile disease that can be accompanied by rashes while adults may experience fever that is characterized by headache, myalgias, arthralgia and rashes (Guzman and Kouri, 2004). However, children and female with chronic disease such as asthma and diabetes appear to be at greater risk of DHF/DSS (Guzman and Kouri, 2004).

2.2.4 Chikungunya

2.2.4.1 Chikungunya virus

Chikungunya infection is an epidemic viral disease responsible for significant global health problem frequently in Africa and Asia (Strauss and Strauss, 1994). The disease is caused by CHIKV of genus Alphavirus in Togoviridae family (Strauss and Strauss, 1986). CHKIV has 11.8 kb long, positive sense single stranded RNA genome with two open reading frames encoding for non-structural and structural polyproteins. Non-structural polyprotein is processed to four non-structural proteins (NsP 1-4) and structural polyprotein is processed to form C, E3, 6K, E2and E1 proteins (Fig. 3) (Strauss and Strauss, 1994). There are three CHKV genotypes with different antigenic determinants including, Asian, West African and Indian Ocean (Eastern/ Central Africa) groups (Schuffenecker *et al.,* 2006).

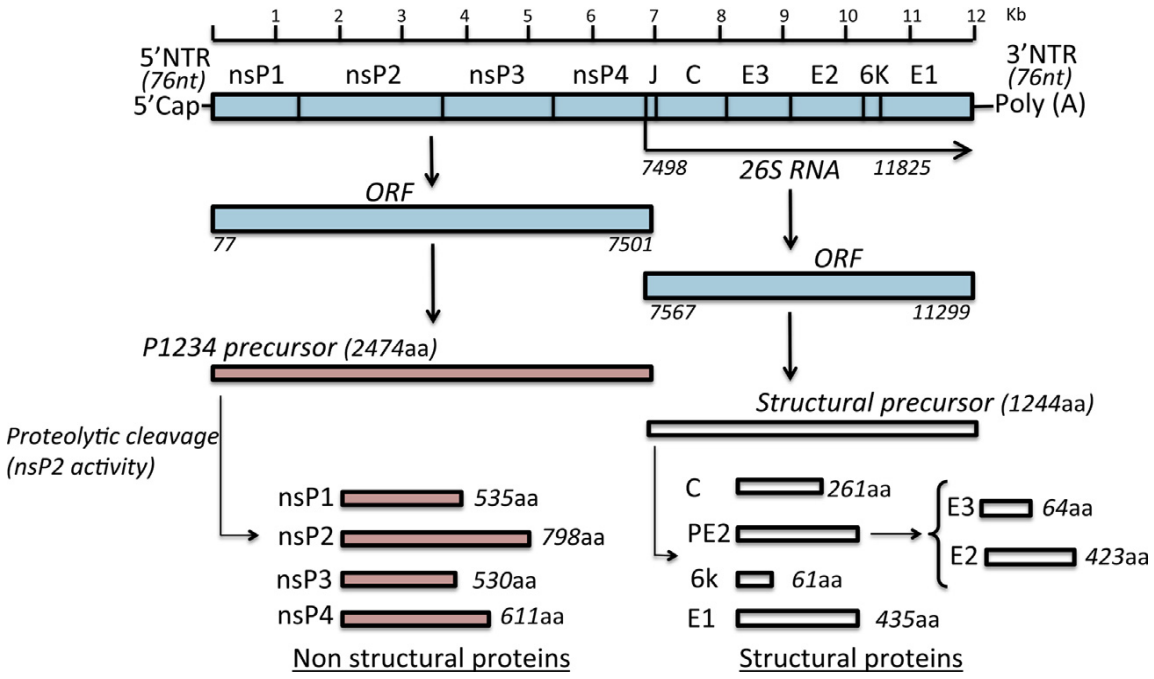

Figure 3: The organization of CHIKV genome. Two open reading frames 5' cap, 3'poly (A) tail and gene products. source: Solignat *et al.,* (2009).

2.2.4.2 Chikungunya

Chikungunya virus infection has two stages, the acute illness and persistent arthropathy (chronic illness). Win *et al.* (2010) observed high fever within 4-7 days, polyarthalgia, backache, headache, fatigue and at lower rate the patient experienced digestive disorders in acute disease. Rhematoid arthritis, spondylarthopathy, fever, fatigue, headache neuropathic pain syndrome, rashes are among the symptoms in persistent arthropathy (Win *et al.,* 2010). Increased age, increased persistent symptoms and viral load are host and virus factors that have been associated with longer duration of illness (Moro *et al.,* 2012). High viral load result into strong early production of ant-viral Ig G hence proposed to protect against chronic long-term CHIKV effect (Moro *et al.,* 2012).

During epidemics human serve as reservoir host for CHIKV while during inter-epidemic period, monkey, rodents and birds are reservoirs (Rezza *et al.,* 2007). There are two transmission cycles, sylvatic in Africa involving transmission between forest mosquitoes and non-human primates with probabilities of spillover to nearby village population and urban human-mosquito-human transmission (Rezza *et al.,* 2007). *Aedes aegypti* and *Ae. albopictus* are main vectors in transmission of CHIKV. Before 2006, *Ae. aegypti* was considered to be principal vector in transmission of CHIKV (Thiberville *et al.,* 2013b). Surprisingly, *Ae. albopictus* was recently identified as a second major vector (Thiberville *et al.,* 2013b). Adaptation of the virus to *Ae. albopictus* was due to adaptive mutation in single nucleotide in E1 gene of CHIKV which assist enhanced viral uptake, replication and transmission (Tsetsarkin *et al.,* 2011). However, in some places *Ae. albopictus* has shown remarkable adaptation to peri-domestic environments and reported to displace *Ae. aegypti* hence become essential vector in CHIKV and DENV transmission (Thiberville *et al.,* 2013b).

Following Chikungunya first outbreak described in 1952 in Makonde plateau along border between southern Tanzania and Mozambique, several other outbreaks have been reported worldwide (Thiberville *et al.,* 2013b). However, in Tanzania the disease is under reported and sometime misdiagnosed as malaria. Huge Chikungunya fever outbreak was reported in Kenya in 2004 that increased global concern of the disease. More than 1000 cases have been reported among European and American travelers from infected areas (Thiberville *at al.,* 2013a). In addition, several CHIKV outbreaks have been reported in 2011-2013 in DRC Congo, India and Philippines which arouses again global concern of this emerging and re-emerging viral disease. Recently, there was a dramatic increased distribution of CHIKV vectors, hence, increased potential to CHIKV cases in future which will not only re-emerge in areas where it has already been isolated but also to newer areas for example the outbreak occurred which in Italy (Thiberville *et al.,* 2013b).

2.2.4 Diagnosis of mosquito-borne viruses

Clinical signs are mostly the used diagnostic methods in most of developing countries; however, they do not provide a clear cut for the disease since a lot of diseases manifest same clinical signs (Chipwaza *et al.,* 2014b). Virus culture remain to be gold standard tool in diagnosis of most viruses however, the technique consumes a lot of time and also needs well equipped facilities. Various serological methods such as antibody detection as well as antigen detections have been developed. In recent years, numbers of molecular techniques (PCR) have been important tools in detection of viral genome. Molecular tools are rapid, sensitive and found to be useful in both entomological surveillance as well as molecular epidemiology of diseases (Guzman and Kouri, 2004). But they are expensive and not available for clinical management.

2.2.5 Management of Rift Valley fever, dengue and Chikungunya

Chikungunya disease is self-limiting, neither has specific treatment nor vaccine. Resting and use of acetaminophen or paracetamol to relieve fever and ibuprofen, naproxen or other

non-steroidal anti-inflammatory agent to relieve the arthritic component used as supportive treatments for patient with Chikungunya (WHO, 2009). Drinking plenty of fluids to replenish fluid lost from sweating, vomiting highly advisable to the patient (WHO, 2009). Prevention of the disease also needs individual prevention from Aedes mosquito bite.

There is no specific treatment develop for RVF. Numbers of vaccines have been developed to prevent the disease. The first vaccine, still in use for animals was developed by Smithburn (Smithburn, 1949). Clone 13, is a second vaccine used in RVF prevention found to be avirulent due to a large deletion in the NSs protein (Muller *et al.,* 1995). In addition, another attenuated MP12 vaccine obtained from the virulent Egyptian strain (Caplen *et al.,* 1985). Recently, MP12 is still being developed as a veterinary and human vaccine. However a person's chances of becoming infected can be reduced by taking measures to decrease contact with blood, body fluids, or tissues of infected animals for case of RVF and protecting themselves against mosquitoes and other blood sucking insects (Chengula *et al.,* 2013).

Management of patients with dengue infections depends on the phase of illness including, febrile phase, leakage phase and convalescence phase. The febrile phase is maintained through reduction of fever with paracetamol, and promotion of food, drinks intake and oral rehydration solution (Kalayanarooj, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The present study was conducted in Kilombero and Ulanga districts of Morogoro Region, South-eastern Tanzania. Kilombero district is coordinating 8. 25° S and 36. 4167° E. The district is located at an elevation of 604 m above sea level and extends from middle to far south-west of Morogoro Region. It is bordered with Morogoro Rural district to the east and Kilosa district to north-east. The north and west borders are shared with Mufindi and Njombe districts of Iringa Region while it shares the border with Songea Rural of Ruvuma Region in the south and Ulanga district in south-east. Kilombero district has five administrative divisions, Ifakara, Mang'ula, Kidatu, Mlimba and Mngeta. The main ethnic groups of Kilombero district are Wapogoro, Wandamba, Wabena and Wambunga. Ulanga district coordinating in 9.0° S and 36.6667° E. The district is located at an elevation of 1009 m above sea level covering 24 460 square kilometres of which 4927 square kilometres are in forest reserve. The district is bordered to the north and west by Kilombero district, to the east by Lindi Region and to the south by Ruvuma Region. Ulanga district consists of five administrative district divisions. Of five divisions, Mahenge is situated in the mountainous portion, and the remaining four divisions. (Mwaya, Lupilo, Mtimbira and Malinyi) are in lowlands. The main ethnic groups of Ulanga district are Wapogoro, Ngindo and Ndamba. Mosquito were collected in Ifakara (Kilombero district) and Mahenge and Mwaya (Ulanga district) (Fig. 4).

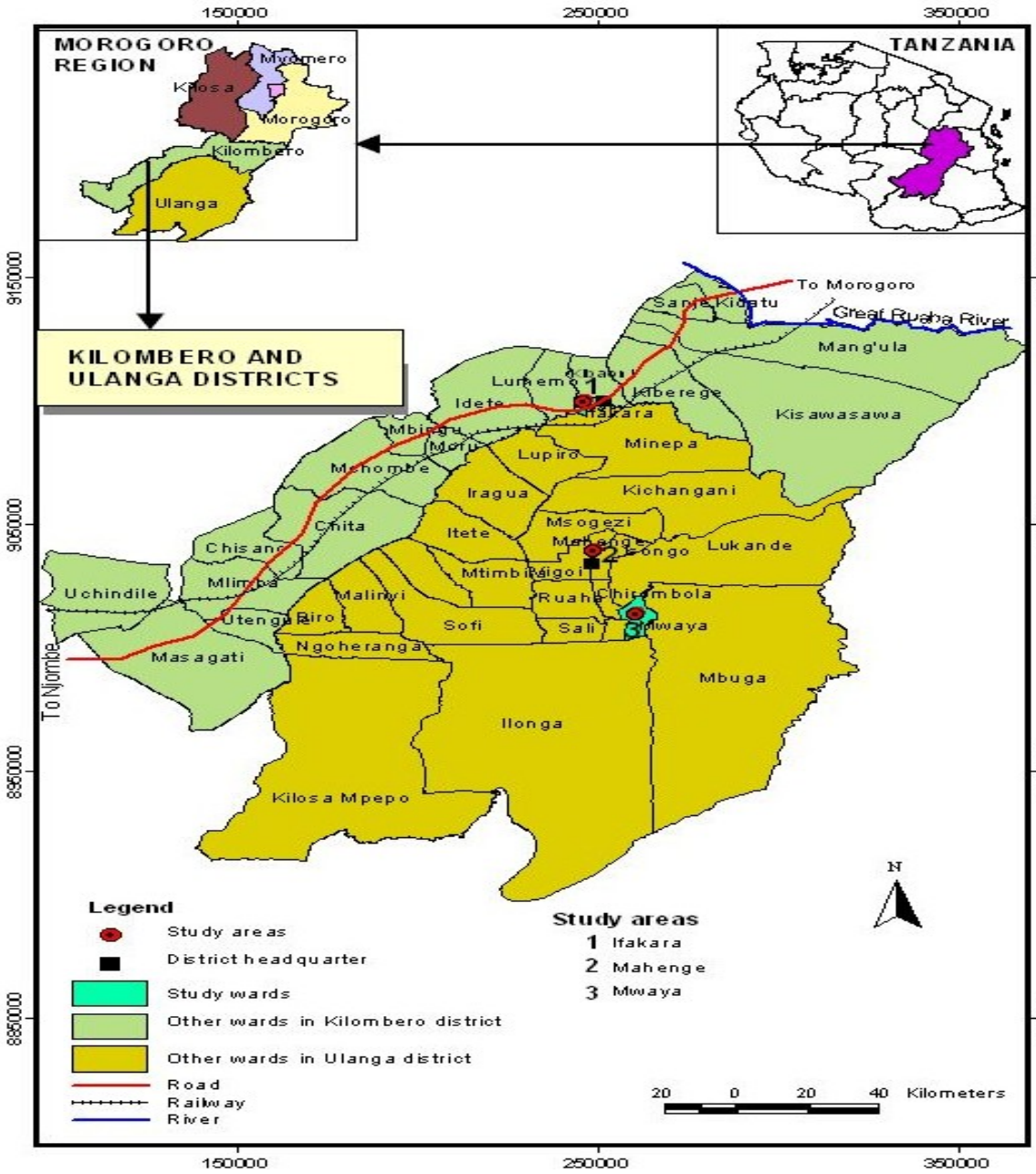

Figure 4: Mosquito sampling sites in Kilombero and Ulanga districts.

3.2 Mosquito Collection

Mosquito collection was carried out at the beginning of dry season in 2015. Biogent sentinel traps and battery-powered aspirators were used to collect mosquitoes. Biogent sentinel traps with CO_2 attractant were set during day and night in different habitats for five consecutive days per site. The battery-powered aspirators were set and retrieved between 5:00 and 19:00 in both outdoor and indoor areas for five collective days. Adult mosquitoes were collected from garages, around temporary ponds, around rice and banana

plantations, tree sheds, in houses and hospital surroundings (Fig. 5). Larvae were collected from different breeding sits such as, tree hole stagnant water, tyres, stagnant pound water and other collective plastics by using Pasteur pipettes. Larvae were collected during day time and allowed to hatch into adults in hatching bottles. The habitat characteristics were recorded during study period for each site surveyed in a mosquito collection form (Appendix 1).

3.3 Mosquito Identification

All mosquitoes collected were immediately identified to genus and species level with the aid of a light microscope based on morphological identification keys (Edwards, 1941). Identified mosquitoes were pooled into groups of 20 based on their species, site of collection and whether they were blood-feed or not. Afterwards, RNA later was added into mosquitoes to preserve the integrity of RNA. Mosquito were then transported to Sokoine University of Agriculture for screening of mosquito-borne viruses.

3.4 Laboratory Analysis

3.4.1 RNA Extraction

Viral RNA was extracted from Aedes mosquitoes using Viral RNA Mini QIAamp kit (Qiagen, Hilden, Germany), as per manufacturer's instructions (Appendix 2). Briefly, samples were lyses with lysis buffer, followed by protein precipitation with ethanol. The lysate was passed through silica-loaded QIAamp Mini spin column followed by washing of RNA. The viral RNA was eluted and stored at -20 °C until use.

3.4.2 Detection of Viruses in Mosquitoes

3.4.2.1 Detection of Bunyavirus, Flavivirus and Alphavirus.

To detect viruses at genus level, extracted RNA was first converted into cDNA. Briefly, a total of eight µl of RNA were mixed with one µl of random hexamer and one µl deoxyribonucleotide triphosphates (dDNTP) were combined in a PCR tube and placed in a

thermal cycler programmed at 65°C for five minutes to denature the sample followed by cooling the mixture at 4 °C for one minute. To the tubes, the following components were added: two µl of 10x reverse transcriptase buffer, four µl of 25 $MgCl_2$, two µl of 0.1 MDTT, one µl of RNase Out inhibitor (Invitrogen, Carlsbad, and California, CA) and one µl of SuperScript III reverse transcriptase(Invitrogen, Carlsbad, California, CA) and incubated in the thermocycler at the following conditions: 25° C for 15 min, 50° C for 50 min, 85° C for 15 min and 4° C hold temperature. One µl of RNase H was then added and volumes adjusted to 20 µl final volumes for reaction which was then incubated at 37° C for 20min then 10° C. Product was then used for various PCR amplifications using primers targeting virus genera (Bunyavirus, Flavivirus and Alphavirus) (Table 1).For genus amplification PCR, one µl of cDNA template, 10 µl of PCR mix, one µl of forward and reverse primers were added with seven µl of nuclease free water to a total of 20 µl. Amplification was done at denaturation temperature of 95° C for 15min followed by 40 cycles of 95° C for 30 seconds, 55° C for 60 seconds and 72°C for 30secondsfollowed by a final extension at 72° C for 10min and final hold of 4° C.

3.4.2.2 Detection of RVFV, DENV and CHIKV

Virus specific RT-PCR was done using already synthesized cDNA samples which reacted positive in generic amplification. The viral specific primers were used to amplify RVFV, DENV and CHIKIV in mosquitoes. The RVF1/ RVF2 targeting glycoprotein M gene in RVFV, D1/D2 primers amplify the structural protein of DENV and the CHIK 3 F/ CHIK 3 R primers which amplify CHIKV (Table 1).

3.4.4 Gel Electrophoresis and Visualization of PCR Product

PCR products were separated by electrophoresis, 1.5% of agarose gel, 0.5% of Tris acetic acid and two µl of GelRed (Phenix, Candler, USA) were used. Five µl of RNA sample, one µl of 6x loading dye and six µl of one kb DNA maker (Promega, Fitchburg, CA) were loaded and run at 100 volts for 40min. The agarose gel was then visualized through

ultraviolet fluorescence light by using gel documentation system (EZ Gel Doc, BioRad, USA).

3.5 Data Analysis

Microsoft office Excel 2007 program (Microsoft Corporation, Washington, USA) was used to calculate proportion of mosquito diversities.

Table 1: List of primers used for detection of mosquito-borne viruses

Virus	Target gene	Primer	Sequence 5'—3'	Positioning	Size(bp)
Alphavirus	NSP4	VIR2052 F	TGGCGCTATGATGAAATCTGGAATGTT	6971-6997	
		VIR2052R	TACGATGTTGTCGTCGCCGATGAA	7086-7109	138
Flavivirus	NSP 5	FU1	TACAACATGATGGGAAAGAGAGAGAA	9007-9032	
		CFD2	GTGTCCCAGCCGGCGGTGTCATCAGC	9308-9283	276
Bunyavirus	nucleocapsid	BCS82C	ATGACTGAGTTGGAGTTTCATGATGTCGC	86-114	
		BCS332V	TGTTCCTGTTGCCAGGAAAAT	309-329	243
RVF	GlycoproteinM	RVF1	GACTACCAGTCAGCTCATTACC	777-798	
		RVF2	TGTGAACAATAGGCATTGG	1309-1327	550
CHIKV	5'NTR	CHIK3F	CACACGTAGCCTACCAGTTTC	14-112	
		CHIK3R	GCTGTCAGCGTCTATGTCCAC	14-112	98
DENV	Structural protein	D1	TCAATATGCTGAAACGCGCGAGAAACCG	38-65	
		D2	TTGCACCAACAGTCAATGTCTTCAGGTTC	455-483	445

Source: Ochieng *et al.* (2013).

3.6 Ethical Consideration

Ethical clearance to conduct this study was obtained from the Medical Research Coordinating Committee (MRCC) of the Tanzania National Institute for Medical Research and provided with certificate number NIMR/HQ/R.8a/Vol.1x/194 (Appendix 3). districts, village leaders and house residents were sensitized and asked for their permission before installation of mosquito traps in their houses or premises.

CHAPTER FOUR

4.0 RESULTS

4.1 Mosquito Diversity, and Ecology

In this study, adult and larvae of mosquitoes were collected at different sites in Ifakara, Mahenge and Mwaya. A total of 1351 mosquitoes were collected of which 43 (3%) were adult mosquitoes collected using a BG traps, 31 (2%) were larvae collected by dipping techniques and allowed to hatch into adults and 1277 (95%) were adult mosquitoes collected using a backpack aspirators. A total of 98 (7.4%) of adult mosquitoes collected were blood-fed. Based on morphological identification keys, a total of three genera including Mansonia*,* Aedes and Culex were identified. Furthermore, a total of 12 species of Culicinae were identified (Table 2). Mwaya was found with the highest mosquito diversity compared with Mahenge and Ifakara (Table 2). Culex was found to be the most dominant genus representing 89%, followed by Aedes (10.1%) and the least was Mansonia (0.8%) (Table 3). However, the relative abundance of the different mosquito genera (Aedes, Culex and Mansonia) varied according to habitats (Fig. 5).

Table 2: Relative abundance of mosquito species in Mwaya, Mahenge and Ifakara

Species	Mwaya	Mahenge	Ifakara
Cx. quinquefasiatus	250	475	428
Cx. cinereus	16	25	0
Cx. tigripes	1	0	0
Cx. duttoni	2	0	0
Cx. pipiens	1	2	0
Cx. eretmopodites	0	1	0
Cx. uranotaeniahenrardi	1	0	0
M. africanus	7	1	1
M. uniformis	2	1	0
Ae.aegypti	5	5	118
Ae. simpsonie	3	1	2
Ae. pemboensis	3	0	0
Total	**291**	**511**	**549**

Table 3: Relative abundance of mosquito genera in Mwaya, Mahenge and Ifakara

Site	Geographical coordinates	Elevation in meters	Culex	Aedes	Mansonia
Mwaya	-0260600 -9035337	1177	271	11	9
Mahenge	-0267440 -9132139	3262	503	6	2
Ifakara	-0244625 9100008	855	429	120	0
Total (%)			**1203 (89.1%)**	**137 (10.1%)**	**11 (0.8%)**

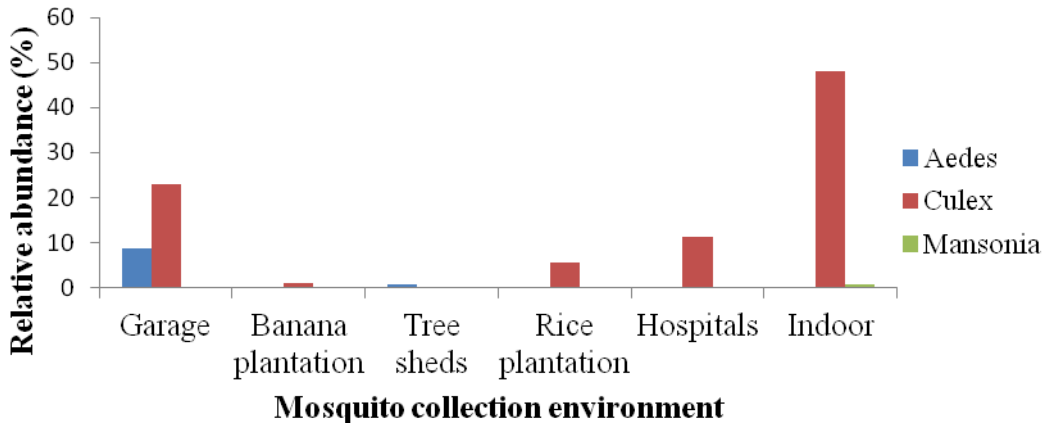

Figure 5: Mosquitoes diversity with different environmental characteristics

4.2 Molecular Detection of Mosquito-borne Viruses

The presence of mosquito-borne viruses in Aedes mosquitoes was detected by RT-PCR, as previously described by Ochieng *et al*. (2013). The expected PCR products were 243, 276 and 138 bp for Bunyavirus, Flavivirus and Alphavirus, respectively. Out of 12 blood-fed Aedes mosquito pools tested, single and multiple infections with Bunyavirus, Alphavirus and Flavivirus were observed (Table 4). Mosquito pools that were positive for Flavivirus, Bunyavirus and Alphavirus were tested for the presence of DENV, RVFV and CHIKV, respectively. No pool was positive for CHIKV or DENV. However, two pools that were positive for Bunyavirus also tested positive for RVFV (Table 4). The RVFV-positive pools included *Ae.aegypti* and *Ae. pembaensis* collected in Ifakara and Mwaya, respectively (Table 4).

Table 4: Mosquito-borne viruses in Aedes mosquitoes

Location	Mosquito species	Pool number	*Alphavirus*	Flavivirus	*Bunyavirus*
Ifakara	*Ae. aegypti*	1	±[a]	+	-
	Ae. aegypti	2	+	+	-
	Ae. aegypti	3	-	+	-
	Ae. aegypti	4	+	+	-
	Ae. aegypti	5	+	+	-
	Ae. aegypti	6	+	+	-
	Ae. aegypti	7	±	-	+[b]
	Ae. simpsonie	11	-	-	-
Mahenge	*Ae. aegypti*	8	-	+	-
Mwaya	*Ae. aegypti*	9	-	+	-
	Ae. simpsonie	10	-	-	-
	Ae. pembaensis	12	-	-	+[b]

[a]Weakly positive; [b]Pools also tested positive for Rift Valley fever virus.

CHAPTER FIVE

5.0 DISCUSSION

In present study, mosquito diversity in selected sites of Kilombero Valley was investigated. Culex mosquitoes were dominant group comprising 89.1% with the highest number of species among all mosquitoes collected (Table 2 and 3). In addition, Cx *quinquefasiatus* was the dominant species of all collected mosquito species (Table 2). The results were comparable to another study which also found Cx *quinquefasiatus* to be dominant among mosquito species (Calhoun *et al.,* 2007). However, *Cx. pipiens* was found to be dominant species (87%) in Ngorongoro district (Mweya *et al.,* 2015). The higher relative abundance of *Cx. quinquefasiatus* can be explained by the presence of favorable breeding sites in the sampling locations such as houses and garages (Fig. 5). Increased pollution has positive influence on the population of *Cx. quinquefasiatus* which prefer to breed on wet pit latrines, septic tanks, cesspits, drains and canals containing stagnant water polluted with organic compound waste. *Cx. quinquefasiatus* also breeds in pools and disuse wells used for dumping garbages (Hougard *et al.,* 1993). Aedes group was the second most abundant group (10.1%) with high number of *Ae. aegypti* (Table 3). The abundance of *Ae. aegypti* observed could be due to their breeding adaptation in artificial plastics found to contain stagnant water. Among all collected mosquito, 8.8% of Aedes mosquitoes were collected in unused car tyres which were found in most garages around town (Fig. 5).

Mosquito species found in Ifakara, Mwaya and Mahenge are potential vectors of mosquito-borne viruses, since, RVFV was isolated from *Ae. aegypti, Ae. simpsonie, Ae. pembaensis, Cx. quinquefasiatus, M. uniformis, M. africanus* and *Cx. pipiens* (Fontenille

et al., 1998). In addition, *Ae. aegypti* are also important vectors of CHIKV and DENV. Findings from this study showed the close proximity between *Ae. aegypti* and *Cx. quinquefasiatus* with human population due to their adaptation in their breeding site, information which provides vital step for identifying potential areas which might influence host-vector interactions and eventually emergence of mosquito-borne virus epidemics. This study was conducted during the dry season; these areas are likely to be of encouraging conditions for breeding of mosquitoes during rainy season. However, breeding of mosquitoes does not depend only on rain season especially for *Ae. aegypti* and *Cx. quinquefasiatus*.

In the present study, Alphavirus, Bunyavirus and Flavivirus were detected in Aedes mosquitoes. Alphavirus were only detected in Ifakara, but not Mwaya and Mahenge. Flavivirus were detected in Aedes mosquitoes at all three sampling locations including Mwaya, Mahenge and Ifakara. Bunyavirus were detected in Aedes mosquitoes of Ifakara and Mwaya. RVFV was detected in *Ae. aegypti* and *Ae. pemboensis* (Table 4). These mosquitoes were collected in banana farms and garages indicating their close proximity to humans thereby increasing the probability of RVFV transmission. A recent study reported a post-epidemic and transmission of RVFV in livestock population in Kilombero (Sumaye *et al.,* 2013), giving a clue RVF is one of febrile illnesses agent in Kilombero valley. Research conducted in Kilosa has shown the circulation of DENV and CHIKV in children (Chipwaza *et al.,* 2014b).

Results of this study indicate the possibility of existence of other mosquito-borne viruses other than DENV, CHIKV and RVFV. This is due to the fact that CHIKV and DENV could not be detected in Aedes mosquitoes that tested positive for Alphavirus and

Flavivirus. The study showed mix infections of viruses in mosquitoes (Table 4). Therefore, this particular study provides an insight to the health personnel and to government on involving virus diagnoses so as to have clear information on mosquito-borne virus infections. In addition, it also encourages the active disease surveillance in both host and vectors to raise public awareness on intra-endemic infection which is mostly asymptomatic or misdiagnosed for other disease.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In the present study, Culex, Aedes and Mansonia genera were identified. Culex represented the major proportion followed with Aedes and Mansonia genera.

Screening of mosquito-borne viruses in Aedes mosquitoes the present study showed the presence of Alphavirus, Bunyavirus and Flavivirus. However, RVFV but not DENV and CHIKV was detected in Aedes mosquitoes. This indicated the presence of other Flaviviruses and Alphaviruses in Aedes mosquitoes other than DENV and CHIKV.

6.2 Recommendations

i. This preliminary study raises attention to health authorities in Tanzania to focus on testing for the presence of mosquito-borne viruses in febrile patients in order to establish effective preventive and control measures. Health care personnel should take into account mosquito-borne viruses in their differential diagnoses in febrile individuals. This may reduce the misuse of antimalarial drugs and thus lead to better prognosis of febrile individuals.

ii. The study recommends further investigation should be done on mosquito-borne virus in all mosquito species as well as in human population of Kilombero river basin and Tanzania as a whole. This would provide clear information on vector abundance as well as virus load.

iii. The study suggests the application of Molecular epidemiology to viruses and vector competence to have an insight into the dynamics of transmission as well as evolutionary trends of the viruses.

iv. Environmental factors favoring vector abundance were identified such as polluted water bodies for Culex and manmade water collection containers for Aedes .The study recommends education to Kilombero community on proper ways of mosquito control

REFERENCES

Allander, T., Emerson, S. U., Engle, R. E., Purcell, R. H. and Bukh, J. (2001). A virus discovery method incorporating DNase treatment and its application to the identification of two bovine parvovirus species. *Proceedings of the National Academy of Sciences* 98(11): 609 - 614.

Bird, B. H., Khristova, M. L., Rollin, P. E., 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: 2805 - 2816.

Calhoun, L. M., Avery, M., Jones, L. A., Gunarto, K., King, R., Roberts, J. and Burkot, T. R. (2007). Combined sewage overflows (CSO) are major urban breeding sites for *Culex quinquefasciatus* in Atlanta, Georgia. *American Journal of Tropical Medicine and Hygiene* 77(3): 478 - 484.

Caplen, H., Peters C. J. and Bishop, D. H. L. (1985) Mutagen-directed attenuation of Rift Valley fever virus as a method for vaccine development. *Journal of General Virology* 66: 2271- 2277.

Charlwood, J. D., Vij, R. and Billingsley, P. F. (2000). Dry season Refugia of malaria-transmitting mosquitoes in a dry savannah zone of East Africa. *American Society of Tropical Medicine and Hygiene* 6(6): 726 - 732.

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(1): 549.

Chipwaza, B., Mugasa, J. P., Mayumana, I., Amuri, M., Makungu, C. and Gwakisa, P. S. (2014a). Community knowledge and attitudes and health workers' practices regarding non-malaria febrile illnesses in Eastern Tanzania. *Neglected Tropical Diseases* 8(5): 2895.

Chipwaza, B., Mugasa, J. P., Selemani, M., Amuri, M., Mosha, F., Ngatunga, S. D. and Gwakisa, P. S. (2014b). Dengue and Chikungunya fever among viral diseases in outpatient febrile children in Kilosa district hospital, Tanzania. *Journal of Neglected Tropical Diseases* 8 (11): 1371.

Edwards, F. W. (1941). Mosquito of the Ethiopian Region (British Museum). *Natural History*. 491 pp.

Elliott, R. M., Schmaljohn, C. S. and Collett, M. S. (1991). Bunyaviridae genome structure and gene expression current topics in Microbiology and Immunology. Springer-Verlag, Berlin. pp 91 - 142.

Feikin, D. R., Olack, B., Bigogo, G. M., Audi, A. and Cosmas, L. (2011). The burden of common infectious syndromes at the clinic and household level from population based surveillance in rural and urban Kenya. *PLoS ONE* 6: 16085.

Fontenille, D., Traore-Lamizana, M., Diallo, M., Thonnon, J., Digoutte, J. P.and Zeller, H. G. (1998). New vectors of Rift Valley fever in West Africa. *Emerging Infectious Diseases* 4: 289 - 293.

Gebhard, L. G., Filomatori, C. V. and Gamarnik, A. V. (2011). Fundamental RNA elements in the dengue virus genome. *Viruses* 3(9): 1739 - 1756.

Gibbons, R. V. and Vaughn, D. W. (2002). Dengue: an escalating problem. *British Medical Journal* 324: 1563 - 1566.

Gillies, M. T. and de Meillon, B. (1968). The Anophelinae of Africa South of the Sahara (Ethiopian Zoogeographical Region). *Publications of the South African Institute for Medical Research* 54: 1 - 343.

Gulber, D. J. (2001). Human arbovirus infection worldwide. *Annals of the New York Academy of Science,* 951: 13 - 24.

Gubler, D. J. and Rosen, L. (1976). Variation among geographic strains of *Aedes albopictus* in susceptibility to infection with dengue viruses. *American Journal of Tropical Medicine and Hygiene* 25: 318 - 325.

Guzman, M. G. and Kouri, G. (2004). Dengue diagnosis advances and challenges. *International Journal of Infectious Disease,* 8: 69 - 80.

Harbach, R. (2015). Mosquito Taxonomic Inventory. [http://www.mosquito-taxonomic-inventory.info] site visited on 9/9/2015.

Harbach, R. E. (2007). The *Culicidae* (Diptera): A review of taxonomy, classification and phylogeny. (Eds Zhang, Z. Q and Shear, W. A) Linnaeus Tercentenary: Progress in Invertebrate Taxonomy. *Zootaxa* 638(1668): 591 - 638.

Hopkins, G. H. E. (1952). Mosquitoes of the Ethiopian Region I-Larval Bionomics of mosquitoes and taxonomy of Culicine larvae. British Museum (Natural History), London. pp 44 - 47.

Hougard, J. M., Mbentangam, R., Lochouarn, L., Escaffre, H., Darriet, F., Barbazan, P. and Quille've're,' D. (1993). Control of *Cx. quinquefasiatus*by *Bacillus sphaericus:* Result of a pilot campaign in a large area in Equatorial Africa. *Bulletin World Health Organization* 71: 367 - 375.

Ibrahim, M., Aziz, A. and Eid-ul-adha, H. (2008). A post card from UK rift valley fever: the story unfolds to family in Sudan. *Sudanese Journal of Public Health* 3: 5 - 10.

Ikegami, T. and Makino, S. (2011). The pathogenesis of Rift Valley fever. *Viruses* 3(5): 493 - 519.

Kalayanarooj, S. (2011). Clinical manifestations and management of Dengue/DHF/DSS. *Tropical Medicine and Health* 39(4): 83 - 87.

Kirby, M. J., Ameh, D., Bottomley, C., Green, C., Jawara, M., Milligan, P. J., Snell, P. C., Conway, D. J. and Lindsay, S. W. (2009). Effect of two different house screening interventions on exposure to malaria vectors and on anaemia in children in The Gambia: a randomised controlled trial. *Lancet* 374: 998 - 1009.

Krzywinski, J. and Besansky, N. (2003). Molecular systematic Anopheles: from subgenera to subpopulations. *Annual Review of Entomology* 48: 111 - 139.

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 Microbiology Review* 18(4): 608 - 637.

Lwetoijera, D. W., Harris, C., Kiware, S. S., Dongus, S., Devine, G. J., McCall P. J. and Majambere, S. (2014). Increasing role of Anopheles funestus and Anopheles arabiensis in malaria transmission in the Kilombero Valley, Tanzania. *Malaria Journal* 13(1): 331.

Mashauri, F. M., Kinung'hi, S. M., Kaatano, G. M., Magesa, S. M., Kishamawe, C., Mwanga, J. R., Nnko, S. E., Malima, R. C., Mero, C. N. and Mboera, L. E. (2013). Impact of indoor residual spraying of lambda-cyhalothrin on malaria prevalence and anemia in an epidemic-prone district of Muleba, north-western Tanzania. *American Journal of Tropical Medicine and Hygiene* 88(5): 841 - 849.

Mboera, L. E. G, Kramer, R. A., Miranda, M. L., Kilima, S. P., Shayo, E. H. and Lesser, A. (2014). Community knowledge and acceptance of larviciding for malaria control in a Rural district of East-central Tanzania. *International Journal of Environmental Research and Public Health* 11: 5137 - 5154.

Mboera, L. E. G., Mazigo, H. D., Rumisha, S. F. and Kramer, R. A. (2013). Towards malaria elimination and its implication for vector control, disease management and livelihoods in Tanzania. *Malaria World Journal* 4(19): 18 - 20.

McCafferty, P. (1983). Mosquito adult, larva and pupa drawings modified from aquatic entomology. Provonsha, A.V. 42pp.

Mhina, A. D., Kasanga, C. J., Sindato, C., Karimribo, E. and Mboera, L. E. G. (2015). Rift Valley fever potential mosquito vectors and their infection status in Ngorongoro district in Northern Tanzania. *Tanzania Journal of Health Research* 17(4): 1 - 9.

Mohamed, M., Mosha, F., Mghamba, J., Zaki, S. R., Shieh, W. J., Paweska, J., Omulo, S., Gikundi, S., Mmbuji, P., Bloland, P., Zeidner, N., Kalinga, R., Breiman, R. F. and Njenga, M. K. (2010). Epidemiologic and clinical aspects of a Rift Valley Fever outbreak in humans in Tanzania, 2007. *American Journal of Tropical Medicine and Hygien* 83(2): 22 - 27.

Moi, M. L., Takasaki, T., Kotaki, A., Tajima, S., Lim, C. K., Sakamoto, M., Iwagoe, H., Kobayashi, K. and Kurane, I. (2010). Importation of Dengue virus Type 3 to Japan from Tanzania and Cote d' Ivoire. *Emerging Infectious Diseases* 16(11): 17770 - 17772.

Moro, M. L., Grilli, E., Corvetta, A., Silvi, G., Angelini, R., Mascella, F., Miserocchi, F., Sambo, P., Finarelli, A. C., Sambri, V., Gagliotti, C., Massimiliani, E., Mattivi, A., Pierro, A. M. and Macini, P. (2012). Long-Term Chikungunya infection clinical manifestations after an outbreak in Italy: a prognostic cohort study. *Journal of Infectious Diseases* 65: 165 - 172.

Muller R., Saluzzo J.F., Lopez N., Dreier T., Turell M., Smith J. and Bouloy M. (1995). Characterization of clone 13, a naturally attenuated avirulent isolate of Rift Valley fever virus, which is altered in the small segment. *American Journal of Tropical Medicine and Hygiene* 53: 405 - 411.

Mwanziva, C. E., Kitau, J., Tungu, P. K., Mweya, C. N. Mkali, H, Ndege, C. M., Sanga, A., Mtambo, G., Lukwaro, C., Azizi, S., Nyamba, J., Chilongola, J., Magesa, S. M., Shekalaghe, S. and Mosha, F. W. (2011). Transmission intensity and malaria vector population structure in Magugu, Babati district in Northern Tanzania. *Tanzania Journal of Health Research* 13(1): 68 - 78.

Mweya, N. C., 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: 25929.

Nimmannitya, S. (1993). Clinical manifestations of Dengue/Dengue haemorrhagic fever. In: Monograph on Dengue/Dengue Haemorrhagic Fever, Regional Publication No. 22. World Health Organization, Regional Office for South-East Asia, New Delhi. pp. 48 - 54.

Ochieng, C., Lutomiah1, J., Makio, A., Koka, H., Chepkorir, E., Yalwala, S., Mutisya, J., Musila, L., Khamadi, S., Richardson, J., Bast, J., Schnabel, D., Wurapa, E. and Sang, R. (2013). Mosquito-borne arbovirus surveillance at selected sites in diverse ecological zones of Kenya; 2007 – 2012. *Journal of Virology* 10: 140-148.

Olive, M. M., Goodman, S. M. and Reynes, J. M. (2012).The role of wild mammals in the maintenance of rift valley fever virus. *Journal of Wildlife Diseases* 48: 241- 266.

Pépin, M., Bouloy, M., Bird, B. H., Kemp, A. and Paweska, J. T. (2010). Rift Valley Fever virus (Bunyaviridae: Phlebovirus): an update on pathogenesis, molecular epidemiology, vectors, diagnostics and prevention. *Veterinary Research* 41: 61.

Renggli, S., Mandike, R., Kramer, K., Patrick, F., Brown, N. J., McElroy, P. D., Rimisho, W., Msengwa, A., Mnzava, A., Nathan, R., Mtung'e, R., Mgullo, R., Lweikiza, J. and Lengeler, C. (2013). Design, implementation and evaluation of a national campaign to deliver 18 million free long-lasting insecticidal nets to uncovered sleeping spaces in Tanzania. *Malaria Journal* 12: 85.

Rezza, G. Nicoletti, L. Angelini, R. Romi, R., Finarelli, A. C., Panning, M., Cordioli, P., Fortuna, C., Boros, S., Magurano, F., Silvi, G., Angelini, P., Dottori, M., Ciufolini, M. G., Majori, G. C. and Cassone, A. (2007). Infection with Chikungunya virus in Italy: an outbreak in a temperate region. *Lancet* 370: 1840 - 1846.

Russell, T. L., Lwetoijera, D. W., Maliti, D., Chipwaza, B. and Kihonda, J. (2010). Research Impact of promoting longer-lasting insecticide treatment of bed nets upon malaria transmission in a rural Tanzanian setting with pre-existing high coverage of untreated nets. *Malaria Journal* 9: 187.

Sang, R., Kioko, E., Lutomiah, J. Warigia, M., Ochieng, C., O'Guinn, M., Lee, J. S., Koka, H., Godsey, M., Hoel, D., Hanafi, H., Miller, B., Schnabel, D., Breiman R. F. and Richardson, J. (2010). Rift Valley fever virus epidemic in Kenya, 2006/2007: the entomologic investigations. *American Journal of Tropical Medicine and Hygiene* 83: 28 - 37.

Schuffenecker, I., Isabelle, I., Iteman, I., Michault, A., Murri , S., Frangeul, L., Vaney, L. M., Lavenir, R., Pardigon, N., Reynes, J. M., Pettinelli, F., Biscornet, L., Diancourt, L., Michel, S., Duquerroy, S., Guigon, G., Frenkiel, M. P., Bre´hin, A. C., Cubito, N., Despre` s, P., Kunst, F., Rey, F. A., Zeller, H. and Brisse, S. (2006). Genome microevolution of Chikungunya viruses causing the Indian Ocean outbreak. *PLOS Medicine* 3(7): 1058 - 1070.

Sindato, C. Karimuribo and E. Mboera, L. E. G. (2011). The epidemiology and socio-economic impact of Rift Valley Fever epidemics in Tanzania. *Tanzania Journal of Health Research* 13(5): 1 - 6.

Smithburn K.C. (1949). Rift Valley fever: the neuro- tropic adaptation of the virus and the experimental use of this modified virus as a vaccine. *Journal of British. Experimental Pathology* 30: 1 - 16.

Solignat, M., Gay, B., Higgs, S., Briant, L. and Devaux, C. (2009). Replication cycle of chikungunya: a re-emerging arbovirus. *Virology* 393(2): 183 - 197.

Solignat, M., Gay, B., Higgs, S., Briant, L. and Devaux, C. (2009). Replication cycle of Chikungunya: a re-emerging arbovirus. *Journal of Virology* 393: 183 - 197.

Strauss, E. G. and Strauss, J. H. (1986). Structure and replication of the alphavirus genome. In: The Togaviridae and Flaviviridae. (Schlesinger, S and Schlesinger, M.J. Eds) New York: Plenum Press. 35- 90pp.

Strauss, J. H. and Strauss, E. G. (1994).The Alphaviruses: gene expression, replication, and evolution. *Microbiology Review* 58(3): 491 - 562.

Sumaye, R. D., Geubbels, E., Mbeyela, E. and Berkvens, D. (2013). Inter-epidemic Transmission of Rift Valley Fever in Livestock in the Kilombero River Valley, Tanzania: A Cross-Sectional Survey. *Journal of* Ne*glected Tropical Diseases* 7(8): 2356.

Thiberville, S. D., Boisson, V., Gaudart, J., Simon, F., Flahault, A. and de Lamballerie, X. (2013a). Chikungunya fever: A clinical and virological investigation of outpatients on Reunion Island, South-West Indian Ocean. *Neglected Tropical Disease* 7(1): 1 - 13.

Thiberville, S. D., Moyen, N., Maguiraga, L. D., Nougairede, A., Gould, E. A., Roques, P. and Lamballerie, X. (2013b). Chikungunya fever: Epidemiology, clinical syndrome, pathogenesis and therapy. *Antviral Research* 99(3): 345 - 370.

Thiel, H. J., Gould, E. A., Heinz, F. X., Houghton, M., Meyers, G., Purcell, R. H. and Rice, C. M. (2005). *Family Flaviviridae*. In: Virus taxonomy, VIII report of the International Committee on Taxonomy of Viruses (Fauquet, M.C.,Mayo, M.A.,Maniloff, J.,Desselberger, U. and Ball, L.A. Eds). Academic Press, San Diego. pp. 981 - 998.

Tsetsarkin, K. A., Chen, R., Sherman, M. B. and Weaver, S. C. (2011). Chikungunya virus: evolution and genetic determinants of emergence. *Recent Current Opinion in Virology Articles* 1: 310 - 317.

URT (2014). Basic demographic and socio-economic profile statistical tables of Tanzania mainland. National Bureau of statistics.

Weaver, S. C. and Reisen, W. K. (2010). Present and future arboviral threats. *Antiviral Research* 85(2): 328.

WHO (2002). Dengue and dengue hemorrhagic fever. Fact Sheet No. 117. WHO, Geneva. [http://www.who.int/mediacentre/factsheets/f117/en/print.html] site visited on 12/09/2015.

WHO (2009). Guidelines for prevention and control of Chikungunya fever. WHO Geneva. 42pp.

WHO (2014).Dengue outbreak in the United Republic of Tanzania (Situation as of 30 May 2014). WHO, Geneva. [http://www.afro.who.int/en/clusters-a-programmes/dpc/epidemic-a-pandemic-alert-and-response/4155] site visited on12 /09/2015.

Win, M. K., Chow, A., Dimatatac, F., Go, C. J. and Leo, Y. S. (2010). Chikungunya fever in Singapore: acute clinical and laboratory features, and factors associated with persistent arthralgia. *Journal of Clinical Virology* 49: 111 - 114.

Woods, C. W., Karpati, A. M., Grein, T., McCarthy, N., Gaturuku, P., Muchiri, E., Dunster, L., Henderson, A., Khan, A. S., Swanepoel, R., Bonmarin, I., Martin, L., Mann, P., Smoak, B. L., Ryan, M., Ksiazek, T. G., Arthur, R. R. Ndikuyeze, A., Agata, N. N., Clarence, J. and Peters, C. J. (2002). An outbreak of Rift Valley Fever in northeastern Kenya, 1997-98. *Emerging Infectious Diseases* 8: 138 - 144.

APPENDICES

Appendix 1: Mosquito collection form

Country: Collection site:

<table>
<tr><td>Collection No.</td><td colspan="2">Longitude/latitude</td></tr>
<tr><td>State</td><td colspan="2">Locality</td></tr>
<tr><td>TOPOGRAPHY
o Mountain
o Hill
o Valley
o Plateau
o Plain

ENVIRONMENT
o Rain forest
o Evergreen forest
o Village
o Urban
o Swamp-open
o Rice paddy

ENVIRO. MODIFIERS
o Primary
o Secondary
o Palm
o plantation

COL. METHOD

ELEVATION</td><td>LARVAL HABITAT
o Pond lake
o Ground pool
o Flood pool
o Marsh
o Stream margin
o Stream pool
o Pit
o Tree hole
o Fallen leaf

SITE DIMENSION</td><td>WATER
o Permanent
o Semi- permanent
o Temporarily
Ph_____
Conductivity___
Temperature____

WATER MOVEMENT
o Standing
o Slow
o Moderate flow
o Fast

TURBIDITY
o Clear
o Turbid
o Polluted
o Colored

SHADE
o None
o Partial
o heavy</td></tr>
</table>

Appendix 2: Purification of viral RNA (Spin Protocol)

Pre-prepared with carrier RNA, the AVL buffer (560 µl) was added to 140 µl of pre-homogenized whole mosquito supernatant in a 1.5 ml microcentrifuge tube. The mixture was then vortexed for 15 seconds and incubated at room temperature for 10 minutes for complete viral particle lysis followed by centrifugation at 6 000 x g for 10 seconds in order to remove drops in the lid. The mixture was added with 500 µl of absolute ethanol (98%) then mixed by pulse-vortexing for 15 seconds and centrifugation at 6 000 x g for 10 seconds to remove drops from inside lid.

The binding of RNA to the column was done by the addition of 630 µl of sample mixture to the QIAamp Min column placed in two ml collection tube. The mixture was then centrifuged at 6 000 x g for a minute then the collection tube with filtrate was discarded the step which was twice done.

Afterwards, the bounded nucleic acid was washed by adding buffer AW1 to the column and centrifuged at 6 000 x g for one minute. The filtrate was discarded and the column was placed in to a new collection tube. The second wash was done by addition of 500 µl of buffer AW2 and centrifuged at 20 000 x g for three min and the collection was discarded.

Finally, the bounded nucleic acid was eluted into new 1.5 ml microcentrifuge tube by the addition of 60 µl AVE buffer into the QIAamp Min column and centrifuged at 6 000 x g for one minute. The filtrate (viral RNA) was then stored at -20 °C for further amplification.

Appendix 3: Ethical clearance certificate

THE UNITED REPUBLIC OF TANZANIA

National Institute for Medical Research
3 Barack Obama Drive
P.O. Box 9653
11101 Dar es Salaam
Tel: 255 22 2121400
Fax: 255 22 2121360
E-mail: headquarters@nimr.or.tz

NIMR/HQ/R.8a/Vol. IX/1941

Ministry of Health and Social Welfare
6 Samora Machel Avenue
P.O. Box 9083
11478 Dar es Salaam
Tel: 255 22 2120262-7
Fax: 255 22 2110986

09th April 2015

Ms Mariana Shayo
Sokoine University of Agriculture
Faculty of Veterinary Medicine
Department of Veterinary Microbiology and Parasitology
P O Box 3019, MOROGORO

CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: Detection and characterization of mosquito-borne viruses in febrile patients and mosquitoes of Kilombero and Ulanga districts , Tanzania, (Shayo M *et al*), has been granted ethical clearance to be conducted in Tanzania.

The Principal Investigator of the study must ensure that the following conditions are fulfilled:

1. Progress report is submitted to the Ministry of Health and the National Institute for Medical Research. Regional and District Medical Officers after every six months.
2. Permission to publish the results is obtained from National Institute for Medical Research.
3. Copies of final publications are made available to the Ministry of Health & Social Welfare and the National Institute for Medical Research.
4. Any researcher, who contravenes or fails to comply with these conditions, shall be guilty of an offence and shall be liable on conviction to a fine. NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Sites: Kilombero and Ulanga districts, in Morogoro region.

Approval is for one year: 09th April 2015 to 08th April 2016.

Name: Dr Mwelecele N Malecela

Signature
CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

Name: Dr Margaret E Mhando

Signature
Ag CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, SOCIAL WELFARE

CC: RMO
DED
DMO