MOSQUITO ABUNDANCE AND MOLECULAR DETECTION OF ARBOVIRUSES IN KYELA DISTRICT, TANZANIA

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

2015

ABSTRACT

Arboviruses belong to various families of viruses that are transmitted by arthropods, mainly mosquitoes and ticks causing clinical disease symptoms in humans ranging from febrile illnesses to hemorrhagic fevers. A systematic vector surveillance spanning two months and covering five sites in Kyela district was carried out in order to determine mosquito abundance and evaluate the potential role of *Aedes spp* in arbovirus transmission in the study area. Mosquitoes were collected, identified to species levels by using morphological keys, pooled by species and collection sites and screened for arboviruses by RT-PCR. Adult mosquitoes were collected from April to May, 2015 using, three CO_2-baited CDC light traps. Additionally, two mosquito Magnet traps were used as well as human landing collection (HLC) was conducted in Kyela town, Kajunjumele, Ipida, Matema and Njisi villages. A total of 1 830 mosquito were collected and identified. The most abundant mosquito collected were recorded in Kyela town 33% (n=601) followed by Kajunjumele 21.3% (n=391). *Culex quinquefasciatus* was the most collected 40.4% (n=740), followed by *Aedes aegypti* 26.8% (n=480). Out of these, four genera including *Culex* 41% (n=750), *Aedes* 27.2% (n=499), *Mansonia* 16.5% (n=301) and *Anophele*s 15.3% (n=280) representing 9 species were identified and shown to be present in Kyela. *Aedes* mosquitoes were pooled into 24 groups of 20 mosquitoes and among them, nine (37.5%) were positive for both *Alphaviruses* and *Flaviviruses*. Chikungunya viruses were the only virus detected in six positive pools of *Alphavirus* genus (75%) that were collected mostly in the areas where rice cultivation is common. The findings of this study suggest that people from this

region are highly likely to be exposed to arbovirus infections which may represent significant public health concerns. Further studies to define the risk factors, characterize the circulating viruses and elucidate the epidemiological factors are required in order to institute national interventions to prevent and control these infections.

DECLARATION

I, BISIMWA NTAGEREKA PATRICK, 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 no being concurrently submitted for a higher degree award in any other institution.

Signature ……………………………… ………………….

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The declaration is hereby confirmed;

Signature…………………………… ……………………..

Dr. Christopher Jacob Kasanga Date

(Supervisor)

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ACKNOWLEDGEMENTS

First of all, I want to give thanks to Almighty God without whom none of this research would have been possible. His guidance and blessings surrounded me and my environment.

I am sincerely grateful to my supervisor Dr. Christopher Kasanga for all his help and support during the project. His guidance, encouragement and advices since the development of the proposal up to the completion of this study are highly acknowledged. This dissertation would not have been completed without continuous help, assistance and advices from the Sokoine University of Agriculture professors in the department of Microbiology and Parasitology such as Prof. Paul Gwakisa and Prof. Gerald Misinzo.

I am grateful to the scientific guidance and assistance of Dr. J Petrus of the National Institute for Communicable Disease (NICD) South Africa. My sincere thanks go to the Southern African Centre for Infectious Diseases Surveillance (SACIDS) for providing me with the scholarship and funds to undertake this study.

Special thanks are also extended to Prof. Mushagalusa Nachigera Gustave and Prof. Katcho Karume (Chancelor and Dean of the Faculty of Agricultural and Environment Studies of the Universite Evangelique en Afrique respectively) for recommending and supporting my studies abroad.

Finally, I offer sincere appreciation to my brother Eric Munguakonkwa, my wife Nadege Mapenzi and all my young brothers and sisters for keeping me sane, their moral support, prayers and encouragement during this long process.

DEDICATION

This dissertation is dedicated to the Almighty God for free life, Prof. Mark Rweyemamu and his fellow Senior Scientists for their role in improving animal health, human health and livelihoods under the concept of "One Health" in East, Central and Southern Africa.

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ABREVIATION AND ACCRONYMS

%	percentage
˚C	degree Celsius
<	less than
>	great than
µl	Microliter
Ae	*Aedes*
An.	*Anopheles*
bp	Base pair
CDC	Centers for disease control and prevention
cDNA	Complementary deoxyribonucleic acid
CHIKV	Chikungunya virus
CO_2	Carbone dioxide
COI	Cytochrome oxydase subunit 1
CPV	**C**ytophatic vacuole
Cx	*Culex*
DENV	Dengue virus
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immonosorbant Assay
ER	Endoplasmic reticulum
FAO	Food Agriculture Organization
Fig.	Figure
G	Glycoprotein

GPS	Geographic Position System
IgG	Immunoglobilin G
IgM	Immunglobilin M
ISRA	Institut Senegalais de Rechereches Agricoles
Kb	Kilo base
kDa	Kilo Dalton
L	Large
M	Medium
ml	Milliliter
MM	Mosquito magnet trap
Mn.	*Mansonia*
MRCC	Medical Research Coordinating Committee
mRNA	Messenger ribonucleic Acid
NC	Negative control
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
NIMR	National Institute for Medical Research
Nm	Nanometer
NRCC	National Research Coordinating Committee
NS	Non-structural
NSP	Non-structural protein
ORF	Open reading frame
PCR	Polymerase Chain Reaction
PRNT	Plaque Reduction Neutralization Test

qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
RAPD	Randomly amplified polymorphic deoxyribose nucleic acid
rER	Rough endoplasmic reticulum
RNA	Ribonucleic Acid
Rpm	Rotor per minute
RT-PCR	Reverse transcription polymerase chain reaction
RVFV	Rift Valley Fever Virus
S	Small
SACIDS	Southern African Center for Infectious Disease Surveillance
SUA	Sokoine University of Agriculture
TGN	Trans-Golgi network
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization
YFV	Yellow Fever Virus

CHAPTER ONE

1 0 INTRODUCTION

1.1 Background information

Arboviruses (Arthropod-borne viruses) compose a large group of zoonotic viruses that are commonly transmitted to humans mainly by the bite of mosquitoes and less frequently by ticks, which results in disease (Marcos *et al*., 2013). They are classified into Togaviridae, Flaviviridae, Bunyaviridae and Reoviridae families having mostly single-strand RNA genome with spherical morphology and a diameter that ranges from 45-120 nm (Figueiredo, 2007). Once confined to limited geographic areas, several of these viruses have spread well beyond their historically endemic regions to become pathogens of global importance (Gubler, 2002).

The Chikungunya virus (CHIKV), Dengue virus (DENV), Yellow fever virus (YFV) and Rift Valley fever virus (RVFV) are included in the group of these viruses. They are considered to be the most common emerging pathogens transmitted to humans by *Aedes* mosquitoes and cause major disease burdens in tropical and subtropical countries worldwide (WHO, 2011). Most of these viruses are maintained in zoonotic cycles and humans are usually incidental dead-end host with an insignificant role in maintaining the cycle of the virus. They constitute a growing international public health problem for which a licensed vaccine, therapeutic drugs, and effective vector control programs are lacking (Gubler, 2002). They cause clinical syndromes of varying severity in humans and animals, ranging from self-limiting febrile illnesses to life-threatening encephalitis and/or

haemorrhagic fever in humans and overt to severe/fatal disease in animals (Sang and Dunster, 2001). The increase in activities on forested areas worldwide is likely to increase human exposure to sylvatic or forest arbovirus cycles. Tropical areas in particular, with the year round hot and humid conditions, are well suited for maintenance of arboviruses that have the potential to emerge as significant human pathogens (Appawu *et al*., 2006).

Tanzania has had multiple arbovirus outbreaks resulting in economic and public health distress including: Rift valley fever reported for the first time in 1930 followed by periodic epidemics of 10-20 years in 1947, 1957, 1977, 1997 and 2007 (Sindato *et al*., 2011). By the end of 2007, the disease had claimed thousands of cases in ruminants and several hundred human cases (EMPRESS, 2008). Chikungunya fever was reported in 1953 where patients were described to have acute onset of fever associated with rigor headache, joint pain and rash (Robinson, 1955). Some cases of dengue were detected from patients in Dar-es salaam in 2010 (Hertz *et al*., 2012). However, recent increase in the density and distribution of the urban mosquito vector, *Aedes aegypti* as well as the rise in air travel increase the risk of introduction and spread of these viruses in non- endemic regions (Alan-Barrett, 2010). Different species of mosquitoes account for the majority of transmissions of the most important vector-borne diseases; each of these species has unique habitat requirements and feeding behaviors, which can vary greatly, even within a closely related group (Gubler, 1998). Knowledge on the infectious status, vectors competence and the geographical distribution of arboviruses may be needed to study

the epidemiological features, the spread and persistence of arboviruses for the development of control measures and prevention of the diseases.

1.2 Problem statement and justification

Most of the Flaviviridae, Togaviridae, and Bunyaviridae arboviruses of medical importance are from tropical Africa sites which are one of the most affected regions in the world today (Powers *et al*., 2000). Febrile diseases caused by viral infections transmitted by arthropods are common in several districts of Tanzania a malaria endemic country; they cause high rate of morbidity and mortality in human as well as in animals because they are often mis-diagnosed and treated as malaria. Much of this confusion stems from the difficulty of clinical discrimination between different arboviral infections, between arboviral infections and those caused by often hyperendemic *Plasmodium falciparum* (Weller *et al*., 2014). The lack of efficient prophylactic and therapeutic measure make infection with these pathogens a serious public health concern not only in endemic developing countries, but also in many non-endemic industrial countries. No studies have been conducted in Kyela district to identify mosquito diversity and to investigate the presence of the selected arboviruses in their potential vectors. However, their diagnosis is still a challenge because of their clinical similarity to other diseases; in most cases the diseases are underreported. Thus, detection of the presence of virus in their arthropod hosts are important for monitoring of viral activity and provide quantitative information that could be useful for modeling the transmission dynamics. Indeed, findings of this could provide important information for understanding arbovirus disease status and their transmission patterns in region with no history of disease outbreaks in Tanzania.

1.3 Research objectives

1.3.1 Overall objective

The main objective of this study was to determine mosquito abundance and to investigate the arbovirus infection status in selected parts of Kyela district.

1.3.2 Specific objectives:

(i) To determine the mosquito species composition and the abundance of known potential arbovirus vector species present in Kyela district,

(iii) To examine the presence of CHIK, DEN, YF and RVF viral genome in *Aedes* mosquito from Kyela district.

1.3.3 Research questions

(i) What are the mosquito species and other potential arbovirus vectors commonly found in Kyela district?

(ii) What is the prevalence of CHIK, DEN, YF and RVF viral genome in *Aedes* mosquito from Kyela district?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mosquito borne viruses

2.1.1 Chikungunya virus

2.1.1.1 Classification and Description

Chikungunya virus (CHIKV) is an arthropod-borne virus belonging to the *alphavirus* genus of the family Togaviridae that is transmitted by *Aedes* mosquitoes. It causes an acute illness characterized by a sudden onset of high fever, rash and joint pain. The most significant symptom of CHIKV-related disease consists of a painful arthralgia that occurs in almost 100% of patients. Other less common symptoms include tenosynovitis, myalgia, retro-orbital pain, pharyngitis, nausea, vomiting, lymphadenopathy, asthenia and dysgeusia (Borgherini *et al*., 2007). Most infections completely resolve within weeks but there are reported cases of CHIKV-induced arthralgia lasting for months, or even for years, in the form of recurrent or persistent episodes (Cavrini *et al*., 2009). It was first isolated in 1952 in the Makonde Plateau of the southern province of Tanzania (former Tanganyika). The name ‘‘Chikungunya’’ in the Bantu language of the Makonde people refers to the stooped posture due to the frequent and debilitating joint pain induced during chikungunya fever (Enserink, 2006).

2.1.1.2 Virus and genome organization

CHIKV is a single-stranded, positive-sense RNA virus and has a diameter of 60-70nm that comprises of a nucleocapsid enclosed within a phospholipid envelope.

The genome is about 11.8Kb and has 2 open reading frames (ORF), encoding a non-structural polyprotein and a structural polyprotein. The non-structural polypeptide is processed to form 4 non-structural proteins (nsP1, nsP2, nsP3 and nsP4) and the structural polyprotein is cleaved to form protein C, E3, 6K, E2 and E1 (Strauss, 1994). Three phylogenetically distinct groups with distinct antigenic properties, based on differing partial CHIKV E1 protein sequences have been identified namely the Asian phylogroup, West African phylogroup and East, Central and Southern African phylogroup (Power *et al*., 2000).

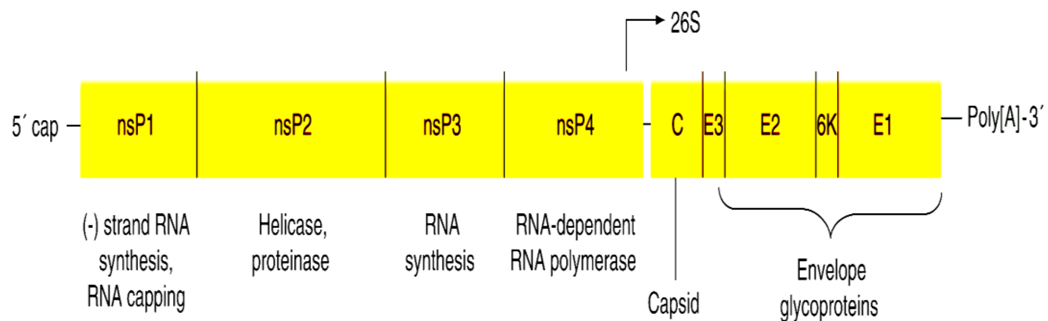

Figure 1: Organization of the chikungunya virus genome, including the nonstructural and structural polyprotein open reading frames, and the 26S or subgenomic promoter. Source: Weaver *et al*., (2012).

2.1.1.3 Replication cycle

CHIKV genome transcription and replication are entirely cytoplasmatic and the virus enters the target cells by endocytosis of clathrin-coated vesicles. The assembled virus particles finally bud through the cell membrane and become enveloped virions. Upon entry Chikungunya virus particle undergoes disassembly, releasing genome RNA in to the cytoplasm of infected cell. The viral genome is then translated from two ORFs to generate the nonstructural (P1234) and structural polyproteins. Early in infection P1234 is cleaved in cis between nsP3 and nsP4 to

yield P123 and ns P4. P123 and nsP4 form an unstable initial replication complex, which is able to synthesize negative-strand RNA (Weaver *et al*., 2012). The polyprotein products nsP1, nsP23 and nsP4 form a replication within virus induced cytophatic vacuoles (CPV I) that are active in negative strand synthesize as well as genomic RNA synthesis, but not in subgenomic RNA synthesis. After complete cleavage to nsP1, nspP2, nsP3 and nsP4, negative-strand synthesis is inactivated and the now stable replication complex switches to synthesis of positive-strand genomic and subgenomic RNA (Solignat *et al*., 2009).

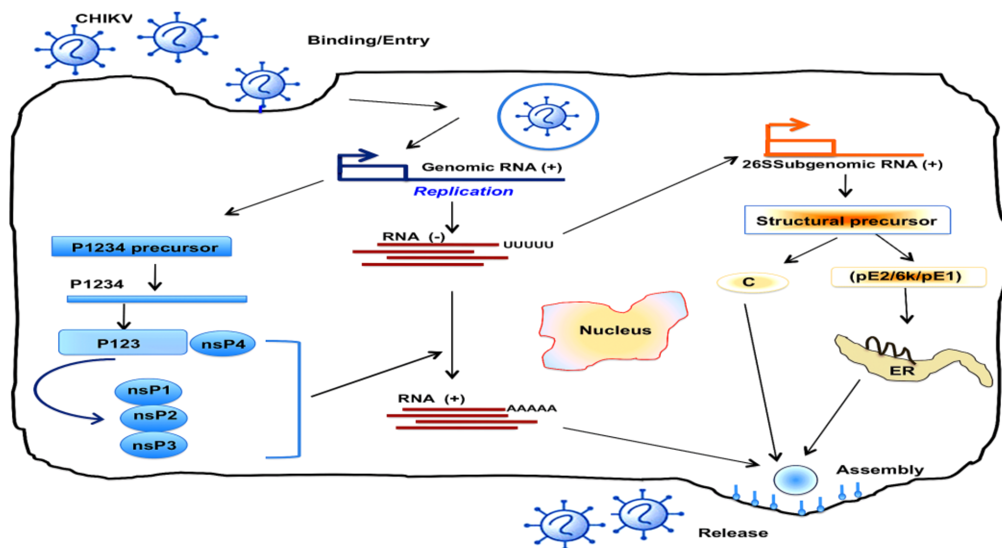

Figure 2: Chikungunya virus replication cycle.

The entry of the virus into the cell is initiated by receptor-binding, followed byclathrin-mediated endocytosis. Fusion to endosomal membrane transports nucleocapsid into the cytoplasm where RNA is released after disambly. Genomic RNA is used for both translation of proteins fron genomic and sub-genomic (26S) RNA, and transcription of nascent (+) RNA via a (-) RNA template. The structural proteins translated from 26S RNA encapasidate nascent genomic RNA before budding from cells and eventual release. Source: Leung *et al*., (2011).

2.1.2.4 Epidemiology

In the period between 2000 and 2005, there were relatively few epidemics with only a few areas reporting outbreaks. By 2000, CHIKV was considered endemic in 23 countries (Powers, 2007). From 2001-2003, Indonesia reported at least 24 outbreaks of CHIKV infection out of which 11 were serologically or virologically confirmed and the remaining based on clinical grounds. This caught global attention and thereafter, epidemics occurred in India, Sri Lanka, Singapore, Malaysia and Italy (Lamballerie *et a*l., 2008; Yergolkar *et al*., 2006). From 2004 onwards, the outbreaks were caused by a variant of the East, Central and Southern African phylogroup (Yergolkar *et al*., 2006). Re-emergence of CHIKV is unpredictable, with intervals between consecutive epidemics ranging from 7 to 20 years, as it is in the case of Indonesia, where cases were reported from 1973 to 1988 and then from 2001 to 2003 (Ligon, 2006). Imported cases into countries with established vectors can result in epidemics as in the case of the 2005 Reunion outbreak. Trade and travel was implicated in the introduction of CHIKV onto Reunion Island which consequently resulted in 266,000 cases (Staples, *et al*., 2009).

2.1.2.5 Transmission cycle

CHIKV can be both endemic and epidemic. It is maintained by two different transmission cycles, specifically sylvatic and human – mosquito (Powers *et al*., 2000). In sylvatic cycles, the main reservoirs for CHIKV are mainly non-human primates, rodents, birds and potentially other vertebrates (Diallo *et al*., 2000). During epidemics, human beings become a reservoir as well. In areas where CHIKV is endemic, there is usually a range of vectors, reservoirs and a local population with

high herd immunity probably due to numerous and continuous transmission (Strauss, 1994). On the other hand, in epidemics, there is usually just one or two vectors, namely *Aedes aegypti* and *Aedes albopictus* and a local population with low herd immunity. For sylvatic cycles, various vectors such as *Aedes furcifer*,*Aedes taylon* and *Aedes luteocephalus* and other species such as *Culex ethiopicus*, *Anopheles coustani and Mansonia fuscopennata* have been implicated (Diallo *et al*.,1999). Otherwise, the usual main vectors in epidemics are the anthroprophilic *Aedes aegypti* and *Aedes albopictus* considered to be traditionally the primary vector.

2.1.3 Dengue virus

2.1.3.1 Classification and description

Dengue viruses (DENV) are arthropod-borne viruses in the genus *Flavivirus* (family *Flaviviridae*) that utilize *Aedes* (Stegomyia) spp., primarily *Ae. aegypti* and to lesser degree *Ae. albopictus*, as vectors for transmission in urban and peri-urban settings. It is caused by any of four related dengue viruses (DENV 1-4). This disease used to be called "break-bone" fever because it sometimes causes severe joint and muscle pain that feels like bones are breaking**;** symptoms, which usually begin four to

six days after infection and last for up to 10 days, may include sudden, high fever, severe headaches, pain behind the eyes, severe joint and muscle pain, nausea vomiting, skin rash, which appear three to four days after the onset of fever (Marcos and Vivaldo, 2013). Morphologically, DENV is a spherical particle of approximately 50 nm in diameter, containing a nucleocapsid of 30 nm surrounded by a lipid envelope. Two structural proteins, the envelope (E) and membrane

proteins (M) are inserted in the lipid membrane (Li *et al*, 2008). The glycoprotein E contains most of the antigenic determinants of the virus and is essential for viral attachment and entry, while protein M, synthesized as the precursor (prM), functions as a chaperone during maturation of the viral particle. The nucleocapsid is composed of the capsid protein (C), a highly basic protein with affinity to RNA, associated to the genome (Kuhn *et al*., 2002).

2.1.3.2 Transmission

Dengue virus can be transmitted from the bite of an infected *Aedes* mosquito. Mosquitoes become infected when they bite infected humans, and can later transmit the infection to other people. Two main species of mosquito, *Aedes aegypti* and *Aedes albopictus*, have been responsible for all cases of dengue transmitted in Mexico. Dengue cannot be transmitted from person to person without a mosquito as the intermediate vector (Gubler, 1998).

2.1.3.3 Genome organization and viral protein expression

DENV viruses have a single stranded RNA genome of positive polarity which is about 11,000 nucleotides long. The RNA carries a single open reading frame encoding a polyprotein that is translated in a cap-dependent manner at the rough endoplasmic reticulum (rER). The viral polyprotein is co- and post-translationally processed into three structural proteins (C, prM, and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Li *et al*., 2008). The amino termini of prM, E, NS1 and NS4B are generated upon cleavage by the host ER signal peptidase in the lumen of the ER, whereas the processing of most of

the other NS proteins and the C-terminus of the C protein is carried out by the viral two-component protease NS2B-NS3 in the cytoplasm of DV infected cells. For the cleavage of the C-terminus of NS1 an unknown ER peptidase seems to be responsible, and a Golgi localized furin protease produces the cleavage of prM at a later state of infection to generate the mature form of M (Izabela *et al*., 2010).

2.1.3.4 Replication cycle

The life cycle of dengue involves endocytosis via a cell surface receptor; the virus uncoats intracellularly via a specific process. The viral non-structural proteins use a negative-sense intermediate to replicate the positive-sense RNA genome which then associates with capsid protein and is packaged into individual virions (Isabela *et al*., 2010). Replication of all positive –stranded RNAviruses occurs in close associatation with virus-induced intracellular membrane structures. The subsequently formed immature virions are assembled by budding of newly formed nucleocapsids into the lumen of endoplasmic reticulum (ER), thereby acquiring a lipid bilayer envelope with the structural protein prM and E. the virions mature during transport through the acid trans-Golgi network, where the prM proteins stabilize the E proteins to prevent conformational changes. Before release of the varions from the hot cell, the matuaration process is completed when prM is cleaved into a soluble pr peptide and virion-associated M by the cellular protease furin. Outside the cell, the virus particles encounter a neutral pH which promotes dissociation of the pr peptides from the virus particles and generates mature infectious virions. At this point the cycle repeats itself (Kuhn *et al*., 2002).

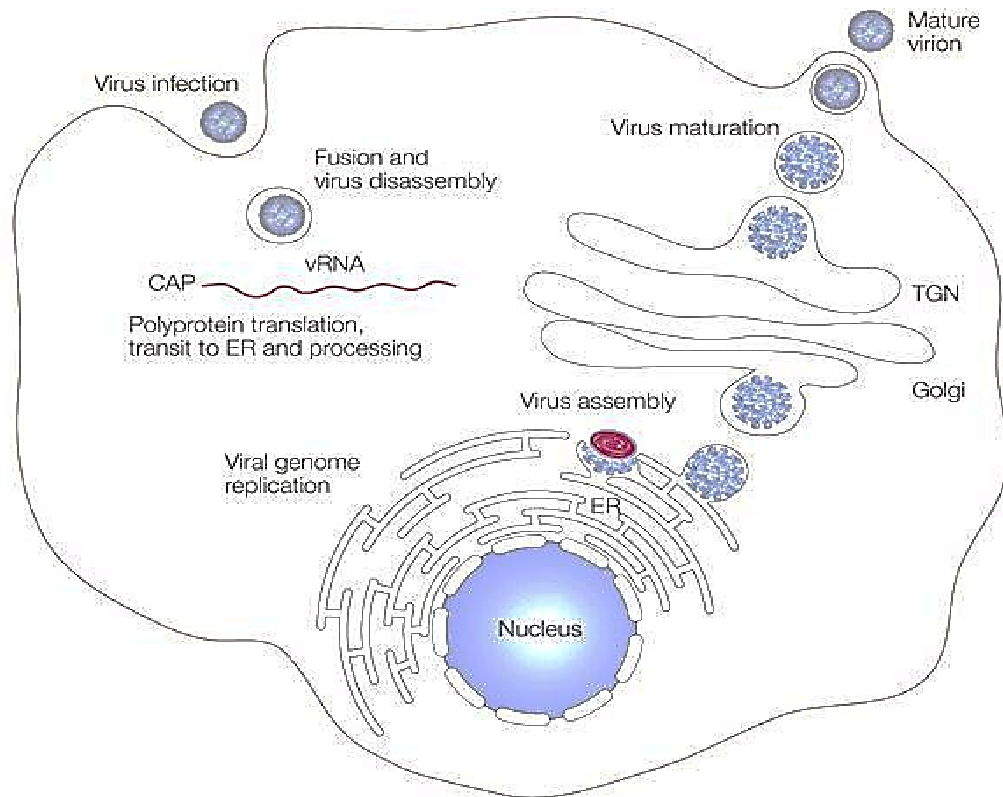

Figure 3: Dengue virus replication cycle.

The dengue virus attaches to the surface of a host cell and enters the cell by a process called endocytosis. Once deep inside the cell, the virus fuses with the endosomal membrane and is released into the cytoplasm. The virus particle comes apart, releasing the viral genome. The viral RNA (vRNA) is translated into a single polypeptide that is cut into ten proteins, and the viral genome is replicated. Virus assembly occurs on the surface of the endoplasmic reticulum (ER) when the structural proteins and newly synthesized RNA bud out from the ER. The immature viral particles are transported through the trans-Golgi network (TGN), where they mature and convert to their infectious form. The mature viruses are then released from the cell and can go on to infect other cells. Source: Kuhn *et al.* (2002).

2.1.4. Rift valley fever virus

2.3.4.1 Classification and description

The Rift Valley fever virus (RVFV), a member of the genus Phlebovirus in the family Bunyaviridae, was first isolated in 1930 during an outbreak in Kenya. Patients who become ill usually experience fever, generalized weakness, back pain, and dizziness at the onset of the illness. However, a small percentage of people infected with RVF develop hemorrhagic fever, which occurs in less than 1%. In animal the disease is recognized by high mortality rates in young animals and abortions in pregnant animals (Pepin *et al.,* 2010).

2.1.4.2 Genome organization

Like all members of the Bunyaviridae family, the RVFV genome comprises three single negative-stranded RNA strands, composed of large (L), medium (M), and small (S) segments (Bird, 2008). All the replication steps occur in the cytoplasm of infected cells and virions mature by budding in the Golgi compartment. The L segment encodes the viral RNA-dependent RNA polymerase. The M segment encodes the structural glycoproteins Gn and Gc, which are expressed as a polyprotein precursor that is processed by cellular proteases during maturation. It has been shown that these structural glycoproteins elicit production of virus-neutralizing antibodies important for protective immunity (Bird, 2008, Pepin *et al*. 2010). The M segment also encodes a nonstructural protein, NSm, which was shown to have an antiapoptotic function and a 78-kD protein whose function is not known yet. The S segment encodes the nucleocapsid protein, N, and a nonstructural protein, NSs, which is recognized as a major virulence factor of the virus by counteracting host innate immunity (Bouloy and Weber, 2010).

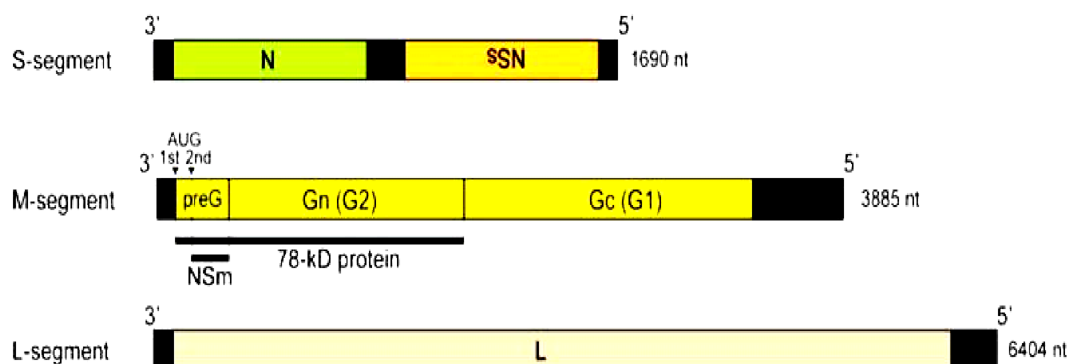

Figure 4: Genome of RVFV showing the three RNA segments.

Small (S), Medium (M) and Large (L) which encode six different proteins. The S segment encodes the N and NSs proteins in an ambi-sense manner. The M segment encodes the NSm, the 78-kDa protein and Gn and Gc, while the L segment encodes the L protein. The 78-kDa and the NSm proteins are synthesized beginning from the first and second AUG of the M mRNA in the preglycoprotein (pre-G) region, which contains five in-frame AUGs. Source: Cheryl and Barr, (2011).

2.1.4.3 Epidemiology and Transmission

RVF is mainly found in countries of sub-Saharan Africa and in Madagascar. An outbreak reported in Saudi Arabia and Yemen in 2000, were the first Rift Valley fever cases identified outside of Africa. These outbreaks occur when areas that are typically dry experience a period of heavy rainfall and/or flooding (FAO, 2008). The mosquito is the vector for RVFV, and epizootics occur during times of heavy rainfall. Normally dry inland depressions known as damboes flood during periods of intense rainfall creating favorable conditions for mosquito breeding. The *Aedes* mosquito is the primary vector for the virus, and it can transmit the virus transovarially to its eggs (Nichol, 2001). The virus has been found to persist in the periods between epizootics in infected mosquito eggs, possibly representing the viral reservoir (Nichol, 2001). Human infections occurs either through direct transmission

from mosquito or through contact with infected tissues or aerosol during the maintenance or slaughter of infected animals (Bird, 2008).

2.1.5 Yellow fever virus

2.1.5.1 Description and transmission cycles

Yellow fever (YF) is a disease caused by mosquito-borne viruses belonging to the family *Flaviviridae,* genus *Flavivirus* and was first isolated from a human case in West Africa in 1927 (Monath and Heinz, 1996). The "yellow" in the name is explained by the jaundice that affects some patients. The clinical disease varies from non-specific, abortive illness to fatal hemorrhagic fever. The incubation period after the bite of an infected mosquito is 3–6 days. Disease onset is typically abrupt, with fever, chills, malaise, headache, lower back pain, generalized myalgia, nausea, and dizziness (WHO, 2000). On physical examination the patient is febrile and appears acutely ill, with congestion of the conjunctivae and face and a relative bradycardia with respect to the height of fever. Three types of mosquito-vectored transmission cycles are recognized for YFV: sylvatic or "jungle" cycle (monkey-mosquito-monkey); intermediate cycle (monkey-mosquito-human); and urban cycle (human-mosquito-human). The "Urban" cycle involves transmission of YFV between humans by *Ae. aegypti*, a domestic vector, which breeds close to human habitation in water and scrap containers including used tires, in urban areas or dry savannah areas (Alan-Barrett,2010).

2.1.5.2 Etiology and genome organization

The causative agent of YF is an arbovirus from the *Flavivirus* genus of the family Flaviviridae. Yellow fever virus is positive-sense, single-stranded RNA virus that

replicate in the cytoplasm of infected cells. Viral particles are 43 nm in size made up of a ribonucleoprotein core and a lipoprotein envelope. It is composed of approximately11000 nucleotides (nt), encoding 10 viral proteins: three structural proteins (capsid [C], pre-membrane [PrM], and the envelope [E]) and seven nonstructural (NS) proteins (NS1-NS2ANS2B-NS3-NS4a-NS4b-NS5-3). The structural proteins are incorporated in released mature virus particles, while the NS proteins responsible for replication remain in infected cells (Marcio *et al*., 2012). The viral envelope consists of a lipid bilayer derived from the infected cell, with dimmers of the envelope (E) protein on the surface anchored at their hydrophobic tails. The E protein is responsible for the initial phases of infection of host cells and is also a principal target for the host's immune response. Yellow fever virus is a single serotype. At the sequence level, five genotypes can be distinguished (three in Africa and two in South America) (Thomas and Monath, 2001).

2.1.5.3. Epidemiology and incidence

Yellow fever occurs in tropical regions of Africa and South America. Fortunately, the virus has never emerged in Asia, and vaccination for travel is not indicated here. Asia is considered vulnerable to the future introduction of the virus, due to the presence of a large susceptible human population and presence of the urban vector, *Aedes aegypti* (Thomas and Monath, 2001). Up to 5000 cases in Africa and 300 in South America are reported annually, but the true incidence is believed to be 10–50 folds higher than the official reports. Between 1990 and 1999, 11297 cases and 2648 deaths were reported in Africa. The largest number of cases was in Nigeria, which suffered a series of epidemics between 1986 and 1994. Epidemics have also occurred in Cameroon (1990), Ghana (1993–1994, 1996), Liberia (1995, 1998),

Gabon (1994), Senegal (1995, 1996), Benin (1996), and Kenya (1992). An epidemic is currently occurring along the border of Liberia and Guinea, an area torn by war with disruption of vaccination and medical services (Thomas and Monath, 2001).

2.1.6 Diagnosis of arboviruses

Preliminary diagnosis is often based on the patient's clinical features, places and dates of travel (if patient is from a non-endemic country or area), activities, and epidemiologic history of the location where infection occurred. The laboratory diagnosis of arboviruses is almost similar to all arboviral diseases. Virus can be isolated or detected by nucleic acid amplification in the acute-phase serum, and various tissues postmortem. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) is the most sensitive method for detecting virus. Histologic techniques can detect viral antigen in autopsy tissues. The most sensitive method of virus isolation is mosquito inoculation, although the uses of C6/36 *Aedes albopictus* cells have more utility for routine isolation (Samuel and Tyagi., 2006). However, serologic diagnosis of DENV infection is problematic because of the extensive cross-reactivity among DENV serotypes and with other *flaviviruses* (Houghton *et al*., 2008). The most useful assay is the IgM-capture enzyme-linked immunobsorbent assay (ELISA), now available commercially. This test, therefore, is useful to detect recent infections, but it is not confirmatory of current infection. Benefits of RT-PCR include high sensitivity, reproducibility and reduced risk of contamination but serological investigations are more commonly done in the clinical setting as they are simpler, faster and cheaper (Thein *et al*., 1992). Immunoglobulin M (IgM) against arboviruses is detectable at day 5 after onset of symptoms via

enzyme-linked immunosorbent assay (ELISA) and can remain detectable for 3 months while IgG is detectable in convalescence and remains so for years (Tyler, 2009).

2.1.7 Treatment and prevention of arboviruses

There is no specific treatment for arboviral infection. Supportive care includes measures to reduce fever, headache, nausea, vomiting, and cerebral edema; management of seizures by anti-convulsants such as Phenobarbital and phenytoin; maintenance of adequate ventilation and blood pressure; and treatment of secondary bacterial infections (Calisher, 1994). Prevention includes vector control, educating the public to avoid high-risk locations, wearing protective clothing, and using DEET-containing insect repellents. There are no commercially available human vaccines for the above mentioned arboviral infections in Africa (Rabia, 1995).

2.1.8 Arboviruses in Tanzania

Tanzania is amongst the countries of the sub-Saharan Africa that has experienced a number of RVF epidemics mostly affecting the northern zone of the country (FAO, 2008). The disease seems to occur following heavy rains and floods that are preceded by periods of long droughts. The latest re-emergence (2006/2007) of the disease among humans and livestock, had expanded to cover different geographical regions , including Manyara, Tanga, Dodoma, Morogoro, Dar es Salaam, Coast, Iringa, Mwanza and Singida, reported cases of RVFV in humans and animals (Nderitu *et al*. 2011).

Recently, four reports of DENV infection in travelers and residents have raised concerns over the occurrence of dengue fever in Tanzania and Zanzibar. (Moi *et al*., 2010). There has been great concern for the likely occurrence of a DENV-3 outbreak in the United Republic of Tanzania. Moi *et al*. (2010) reported two cases of imported DENV-3 infection in Japanese travelers returning from Tanzania, and that same year, a case was reported in a European traveler returning from Zanzibar. A dispatch on May 17, 2010 reported the occurrence of 17 suspected dengue fever cases since April 2010 at a private clinic in Dar es Salaam, mostly among resident expatriates who had traveled to Zanzibar. Finally, on July 27, 2010, the Tanzanian Daily News (a local newspaper) reported the occurrence of 100 acute cases of dengue fever in Dar es Salaam and Zanzibar in febrile patients (Klaassen, 2010). No baseline data from this geographical area is available in the literature on dengue seroprevalence in the general population or on dengue incidence in febrile patients (Moi *et al*., 2010). However, CHIKV was first isolated and described during an epidemic in present-day Tanzania in 1952; since that time, periodic CHIKV outbreaks have been reported across the African continent, (Enserink, 2006), little is known about reports of CHIKV infection in Tanzania since its discovery mostly because, CHIKV infection, like DENV infection, is a diagnosis rarely considered by local clinicians, presumably due in part to a lack of information about disease prevalence (Hertz *et al*., 2012).

No Human cases for yellow fever have been reported from Tanzania. The only evidence for YF came from serological surveys carried out in the 1940s (Tabachnick *et al*. 1985). A low prevalence of children and adults (<5%) at several locations

along the coast from Tanga in the North to Newala in the southeast were seropositive. A survey on Zanzibar conducted in 1951-53 revealed neutralizing antibodies in 2 (4%) of 55 unvaccinated children with no history of having travelled to the mainland (WHO, 2011).

2.2. Mosquitoes

2.2.1 Mosquito description and taxonomy

Mosquitoes are slender, long-legged insects that are easily recognized by their long proboscis and the presence of scales on most parts of the body. Larvae are distinguished from other aquatic insects by the absence of legs, the presence of a distinct head bearing mouth brushes and antennae, a bulbous thorax that is wider than the head and abdomen, posterior anal papillae and either a pair of respiratory openings (subfamily *Anophelinae*) or an elongate siphon (subfamily *Culicinae*) borne near the end of the abdomen (Reinert *et al*., 2004). The time of flight and feeding activity is usually quite specific for most species. Some species are active at night (nocturnal) or twilight (crepuscular) whereas others are active during the daylight hours (diurnal). Mosquitoes belong to the order Diptera (True flies), in the family Culicidae. There are 3 subfamilies; Anophelinae*,* Culicinae *and* Toxorhynchitinae (Harbach and Howard, 2007).

Only *Anophelinae* and *Culicinae* contain mosquitoes that are able to spread diseases. The *Culicidae* family includes 3 539 species classified into two subfamilies (*Anophelinae and Culicinae*) and 112 genera. The subfamily of *Anaphelinae* is comprised up to three genera while the subfamily *Culicinae* has 109 genera segregated into 11 tribes (Harbach and Howard 2007). They are found across almost the entire globe, but the majority are found in the tropics and subtropics.

2.2.2 Habitat and life cycle

The immature stages of mosquitoes occupy a spectrum of aquatic environments. They occur primarily in temporary or permanent bodies of ground water, but a large number of species occupy leaf axils, tree-holes, rock-holes, crab-holes, bamboo internodes, bromeliads and aroids, fruit shells and husks, fallen leaves and spathes, flower bracts, snail shells and pitcher plants. Some utilize artificial containers as well as the normal ground-water habitats. The majority of larvae feed on suspended particulate matter and microorganisms that they extract from the water with filamentous mouth brushes. Other species are obligatory or facultative predators that capture and feed largely on the immature stages of other mosquitoes by means of modified mouth brushes or grasping mandibles or maxillae (Wayne, 2004). Since mosquitoes are delicate insects, they are always found where the air is relatively cool and the humidity is high. Many species live within a few meters of the ground whereas many sylvan species occur primarily in forest canopy. Vertical distribution is largely dependent on feeding preferences. All males and the females of many species feed exclusively on plant liquids, including nectar, honeydew, fruit juices and exudates. Females of numerous species feed on the blood of living animals, but some that are normally hematophagous may produce eggs without a blood meal. Warm-blooded vertebrates are a common source of blood for most species, but many species also attack cold-blooded animals such as snakes, turtles, toads, frogs and other insects, including nymphal cicadas, lepidopterous larvae and mantids (Reinert *et al*., 2004). All mosquitoes undergo complete metamorphosis within their lifecycle, which has four stages of development (egg, larva, pupa and adult) and each stage results in a cast of the exuviae. The life cycle starts by laying eggs on the

surface of the water, either singly (Anopheles, *Aedes*, Orthopodomyia and Culiseta subgenus Culicella), or in batches (*Culex,* Uranataenia, Coquillettidia, and Culiseta subgenus Culiseta). After the eggs hatch, they pass through four larval instars. During this time they feed on detritus, algae, and biofilms (Wayne, 2004).

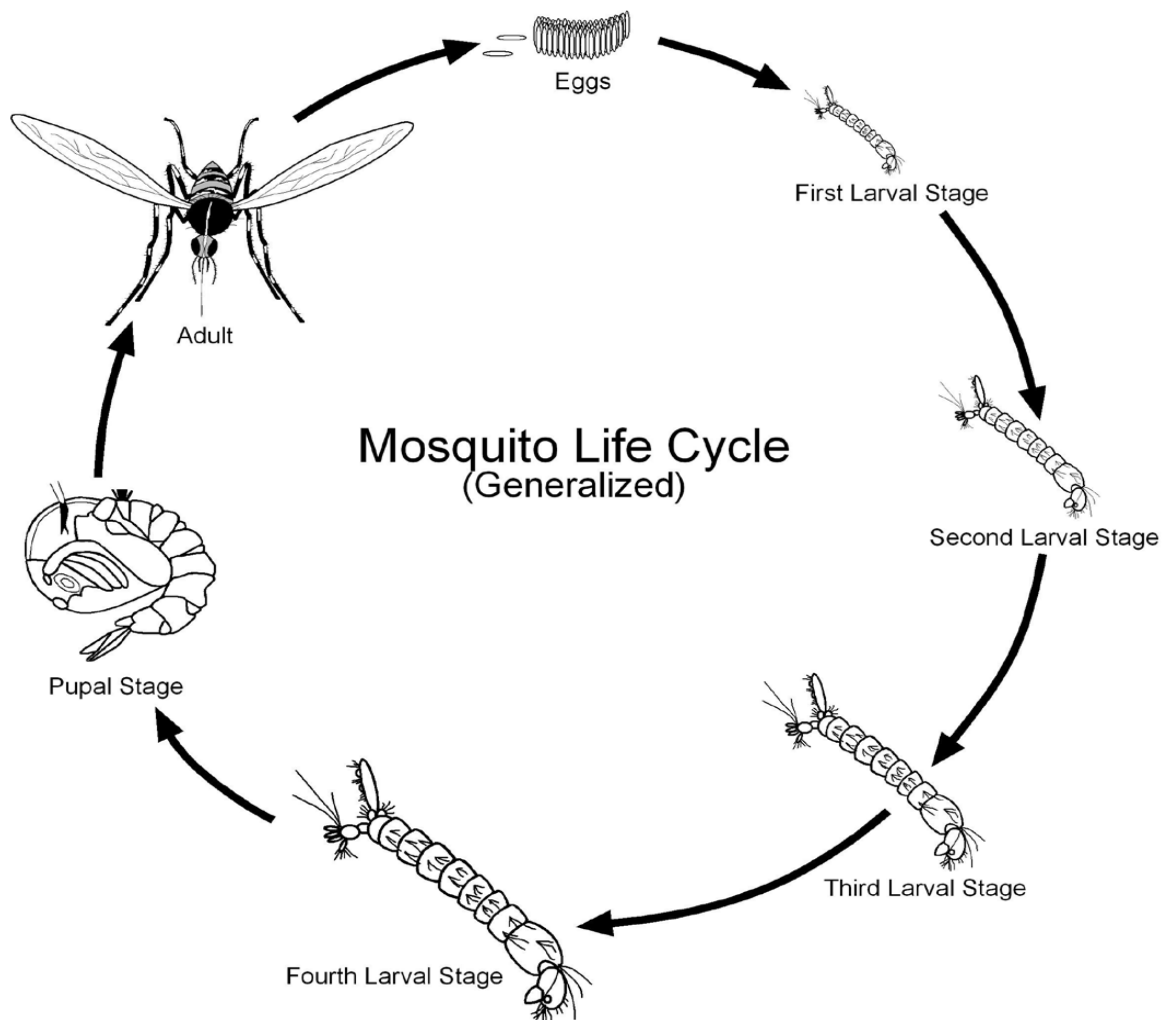

Figure 5: Mosquito life cycle presenting the four life cycle components. An egg stage; four larval stages; a non-feeding pupal stage; and an adult stage.. Source: www.mosquito.org/mosq-biology.htm

2.2.3 Mosquito competence

Numerous factors determine the ability of a mosquito to transmit arboviruses that cause disease. First, a mosquito must be a competent vector. "Vector competence" refers to the ability of a mosquito to acquire an arbovirus from a reservoir host and later transmit the arbovirus to a susceptible host during the act of taking another blood meal. This is dependent on the mosquito being a suitable host in which the arbovirus survives, undergoes essential development, multiples, and eventually reaches and infects the salivary glands. Transmission of an arbovirus occurs by injection of salivary secretions during the act of feeding (Catherine *et al*., 2013).

2.2.4 Techniques for mosquito identification

Many techniques for examining specific behavior, ecology and genetics have been used to reveal, and subsequently identify, morphologically similar species of mosquitoes (Diptera: *Culicidae*). The most commonly used methods are based on the study of morphological as well as molecular DNA based methods.

2.2.4.1 Morphological identification of mosquito species

With an increase number of human cases of mosquito-borne disease causing hemorrhagic fever worldwide, it is essential that identification keys for the mosquito vectors be readily available and used for vector identification. The identification of mosquito species is mostly done basing on observation of morphological features. Several key dichotomous identification pre-imaginal stages and adults *Culicidae* were established by many authors (Rueda, 2004; Gillies and Meillon, 1968). However, this technique can be problematic in the reason that diagnosis

morphological features are often damaged during mosquito collection or their storage. Furthermore, the morphological characteristics to identify intact adult specimens often vary, thus little between species that usually only experienced mosquito taxonomists are able to distinguish mosquito species reliably (Bortolus, 2008).

2.2.4.2 Molecular DNA based methods

DNA analysis provides a more accurate way of identifying species and the use of molecular data, in combination to morphological methods, has resolved some long-standing taxonomic questions (Gang *et al*, 2012). Chromosome differences are a commonly used means of identification of anopheline species, but the technique cannot be applied to all specimens, it is either sex or developmental stage specific. Considerable effort has been expended in finding alternative means of specific identification of mosquitoes, especially to distinguish between sibling species that may differ in their vectorial capacity (Gao *et al*., 2004). Allozyme electrophorese requires fresh or frozen material. Cuticular hydrocarbon analysis using high-performance gas chromatograph has not proved to be diagnostic and requires expensive equipment (Sharpe *et al*., 1999). Three DNA-based approaches have been utilized for mosquito species identification: hybridization assays based on species-specific sequence, randomly amplified polymorphic DNA (RAPD) and tests based on known variable regions. The second and third options involve the polymerase chain reaction (PCR) which are useful and suitable for the identification of specimens of mosquitoes collected at any stage of development and species that are morphologically very similar or indistinguishable (Gang *et al*, 2012). In addition,

many studies have since then demonstrated that the COI gene is a valid molecular tool for identifying mosquito species (Kumari *et al*., 2010).

2.2.4.3 Mosquito diversity in Tanzania

Fewer studies have been conducted for mosquito fauna in Tanzania; the few studies are mostly focused on malaria vectors. Of the 45 species of anopheline mosquitoes recorded in the country, 11 have been implicated as malaria vectors. The three species which, are most commonly involved in malaria transmission in Tanzania, are *Anopheles gambiae* s.s. Giles, *An. arabiensis* Patton and *An. funestus* Giles (Gillies & Meillon, 1968). However, both *Aedes aegypti aegypti* and *Ae. aegypti formosus* occur in East Africa. Genetic and disease-transmission studies provide strong evidence for the specific distinctness of these sub-species. Occasional cases of suspected dengue have occurred in Kenya but these were not confirmed by virus isolation and PCR. Presumably, *Ae. aegypti* transmitted the virus. Outbreaks of yellow fever in East Africa, however, have not been transmitted by *Aedes aegypti*, but by other species such as *Aedes simpsoni* (Failloux, *et al*., 2002).

Despite several decades of malaria research, locality-specific information on the diversity, spatial and temporal distribution of mosquito species and especialy Anopheles *gambiae* complex is still wanting. Species within the complex differ in host biting preference, abundance and vector competence, identification of the mosquito vectors to species level and mapping species distribution in heterogeneous environments is critical (Coetzee, 2004).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

This study was conducted in Kyela district of Mbeya region, located in the South Western corner of the Southern Highlands of Tanzania. The district lies between longitudes 33°41' and 33°30' East of Greenwich and between latitudes 9°25' and 9°40' South of Equator (Fig. 6). Kyela's rainy season is between November and June, with the heaviest rains falling in April and May. The district lies in the flood plains of Lake Nyasa and thus receives heavy rains of about 3000 mm per year. Kyela has a hot and humid climate with a mean daily temperature of 23ºC. The natural vegetation is of tropical savanna forest and grasslands with lagoon vegetation on the swamps and river mouths to the lakes. The study site was chosen to include the coastal areas: Kyela (town), Kajunjumele, Njisi, Ipinda, and Matema because of their location to low altitude (500m) with availability mosquito favourable habitats. Furthermore, their locations on the proximity to the Kyela flood plains and Lake Malawi as important factors which can contribute to arbovirus emergences.

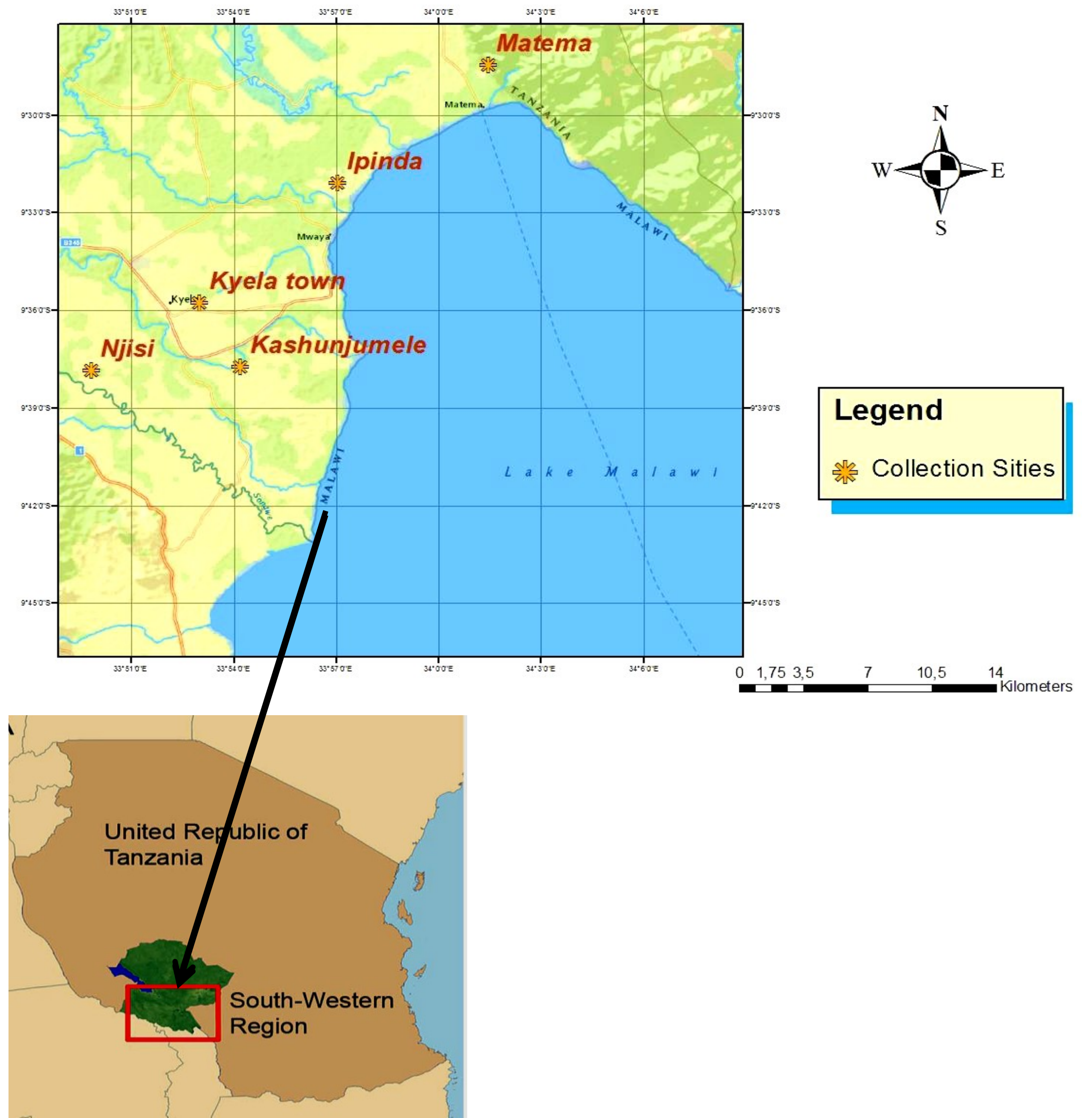

Figure 6: Map of Kyela district showing sampling sites. Source: GPS coordinates.

3.2 Study design and mosquito trapping collection

This study has utilized a cross-sectional panel design and was conducted from April to May 2015, which is rainfall months suitable for mosquitoes breeding; approximately, 1 830 mosquitoes were collected and identified at species level. For the outdoor mosquito collection, the Mosquito Magnet (MM) trap was used, which is battery operated trap and runs on propane gas that is catalytically converted to

produce carbon dioxide (CO2) which attracts the mosquitoes. The trap was run from 1600 hours to 0600 hours outside the houses (outdoor). However, CDC light traps and human landing collection were also used for collection of indoors mosquitoes.

3.3 Mosquito identification

The adult mosquitoes trapped were killed by using alcohol 100%, sorted by genus level, packed in labeled 1.5 ml eppendorf tubes and transported in dry ice to the SACIDS molecular biology laboratory where they were kept in freezer at -20°C before identification. Then after, identification by using a standard morphological identification keys (Gillies and De Meillonm, 1968) under a stereo light microscope, mosquito were pooled (up to 20 mosquitoes per pool) by species and collection sites.

3.4 Molecular analysis

3.4.1 Viral RNA extraction and cDNA synthesis

RNA was extracted from pooled mosquito samples using the QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany) according to manufacturer's recommendations (Appendices 1). Briefly, 140 µl of supernatant were added to 560 µL AVL buffer containing carrier RNA into a 1.5 mL micro-centrifuge tube and mixed by pulse-vortexing for 15 sec followed by incubation at room temperature for 10 min. Protein precipitation was followed by adding 560µL of absolute alcohol mixed by pulse-vortexing for 15 sec. The lysate was then passed through a silice column, and the column was washed twice with 500 µl of washing buffers AW1 and AW2, respectively. Finally, RNAs were carefully eluted by 60 µl of buffer AVE equilibrated temperature and the extracted mosquito RNA was stored in -20˚C at

Southern African Centre for Infectious Diseases Surveillance molecular biology laboratory located at SUA Morogoro before amplification. To convert extracted RNA into cDNA, 4 µl of RNA template was combined into 16 µL of a master-mix containing 4µl of 5XVILO Reaction Mix, 2µl of 10xSuperscript EnzymeMix and 10µl nuclease free water; the whole volume was brought up to 20µL. The tube contents were mixed by vortexing for 15 seconds and the incubated in the thermocycler at the following conditions: 25°C for 10 min, 42°C for 60 min; the reaction was terminated at 85°C for five min and 4°C as hold temperature.

3.4.2 Arbovirus detection by RT-PCR

The cDNA amplicons were used for RT-PCR amplification (Invitrogen One step RT-PCR kit) using primers targeting virus genera or specific arboviruses. A total of 20µL of master mix was prepared containing 10µL of 2xDream Taq green PCR Master Mix (Thermo Scientific), 1µL of both forward and reverse primer, 1µL of cDNA and 7 µL of nuclease-free water up to 20µL. Primers targeting *Bunyavirus*, *Alphavirus* and *Flavivirus* (Table 1) were used to detect the presence of arbovirus specific genera. For a samples which tested positive with genus primers, were tested further with primers that target conserved genes in the specific viruses belonging to the genus in question (Table 2). The PCR cycling performed was the following: an initial denaturation step at 94°C for 15 min, following by 35 cycles of denaturation at 94°C for 30 sec, annealing temperatures at 57°C for 60 sec. each, extension at 72°C for 30 sec. The reaction mixture in each PCR tube was then subjected to a final extension step at 72°C for 10min. The PCR amplification of targeted gene for specific virus in the cDNA was performed in a 25-µl reaction containing: 12.5 µl of

2xDream Taq Green PCR master mix (Thermo Scientific), 0.5 µL each of forward and reverse primer, 2 µl of the cDNA and 9.5 µl of water to top up to 25 µl. Thermal profiles were performed on a GeneAmp PCR system 9700 (Applied Biosystems, USA).

3.4.4 Gel electrophoresis and visualization of RT-PCR products

The RT-PCR products were separated by electrophoresis on 1.5% agarose gel in 0.5% TAE buffer (Serva Elctrophoresis, Heidelberg, Germany). Following amplification, 5µL from each PCR tube containing amplified product and 1µl of blue/orange 6xDNA loading dye (Promega Madison, USA) were loaded onto gels of 1.5% agarose. The gels were stained with 2µL of GelRed, run at 100 volts for 40 minutes and the PCR products were visualized under UV light.

Table 1: DNA sequences of the primers used for detection of arbovirus genera

Virus	**Target gene or protein**	**Primer**	**Sequence (5'→3')**	**Position**	**PCR product size (bp)**	**Reference**
Alphavirus	NSP4	VIR2052F	TGG CGC TAT GAT GAA ATC TGG AAT GTT	6971-6997	150	Esho *et al*., 2005
		VIR2052R	TAC GAT GTT GTC GTC GCC GAT GAA	7086-7109		
Bunyavirus	N Protein	BCS82C	ATC ACT GAG TTG GAG TTT CAT GAT GTC	86-114	251	Bryant *et al*., 2007
		BCS332V	GCTGT TCC TGT TGC CAG GAA AAT	309-329		
Flavivirus	NS5	FU1	TAC AAC ATG ATG GGA AAG AGA GAG AA	9007-9032	220	Kuno *et al*., 1996
		CFD2	GTG TCC CAG CCG GCG GTG TCA TCA GC	9308-9283		

Table 2: DNA sequence of the primers used for screening of specific viruses

Virus	Primers	Sequence (5'-3')	Region, position	Reference
CHIKV	CHIK3F	CAC ACG TAG CCT ACC AGT TTC	5′NTR, 14-112	Smith *et al*., 2009
	CHIK3R	GCT GTC AGC GTC TAT GTC CAC		
DENV	D1 38-65	TCA ATA TGC TGA AAC GCG CGA GAA ACC G	3'UTR, 10520-10541	Lanciotti *et al*.,1992
	D2 455-483	TTG CAC CAA CAG TCA ATG TCT TCA GGT TC	3'UTR, 10674-10694	
RVFV	RVF3	CAG ATG ACA GGT GCT AGC	Gn, 876	Testuro, 2012
	RVF4	CTA CCA TGT CCT CAA T	GlyM, 2817-2840	
YFV	CAG	CGA GTT GCT AGC AAT AAA CAC ATT TGG A	Polypro. 43 - 71	Weidmann *et*
	YF7	AAT GCT CCC TTT CCC AAA TA	Polyprot. 1293-1312	*al*.,2010

3.5 Statistical analysis

The infectious status of mosquito-borne virus in mosquitoes determined by RT-PCR was analyzed with Epi Info 7 software. Chi-square test and Fisher exact tests were used to evaluate heterogeneity of rates among the different villages.

3.6 Ethical consideration

The approval to carry out this study was sought from the ethics review subcommittee of the National Research Coordinating Committee (NRCC) and ethical clearance from National Institute for Medical Research of Tanzania (NIMR). Confidentiality of the study participants was strictly observed by coding the participant's name and its provenance region.

CHATER FOUR

4.0 RESULTS

4.1 Number of mosquitoes collected

During this investigation, a total of 1830 blood-feed mosquitoes were collected from April to May 2015 in Kyela (Town), Njisi, Kajunjumele, Ipinda and Matema villages and were pooled into 91 pools based on the collection sites and genus, including 30; 19; 18; 15 and 9 pools from Kyela (town), Kajunjumele, Njisi, Ipinda and Matema respectively and preserved into silica gel before identification to species levels. Among them, 24 *Aedes* mosquito pools each containing up to 20 mosquitoes were stored at – 20 ˚C for arbovirus screening by using RT-PCR.

4.2 Mosquito distribution and abundance

After morphological identification, a total of 9 species of *Culicidae* which belonged to 4 genera including *Culex, Aedes* , *Anopheles* and *Mansonia* were identified. Among them, 3 species of *Aedes,* 2 species of *Culex, 2* species of *Anopheles* and 2 species of *Mansonia* genus were identified (Table 3). Out of 1830 mosquito collected *Culex* represented the most abundant genus 41% (n= 750), followed by *Aede*s 27.2% (n=499), *Mansonia* 16.5% (301) and *Anopheles* being the minority 15.3% (n=280) (Table 4).

In general, there is significantly different for mosquito abundance between sampling sites ($P < 0.05$; IC 9.54 – 30.57) . The most abundant mosquito collection by site was recorded in Kyela Town 33% (n= 601) followed by Kajunjumele 21.3% (n= 391); Njisi 20% (n=365); Ipinda 16% (n= 293) and Matema village giving the least

collections 10% (n=180) (Fig.7). The most widespread and predominant species sampled in all sites was *Culex quinquefasciatus* 40.4% (n=740), followed by *Aedes aegypti* 26.8% (n=480); *Mansonia uniformis* 15.3% (n= 280); *Anopheles gambaie spp* 14.9% (n=273) and the least overall sampled species were *Mansonia africanus* 1.1% (n=21), *Anopheles funestus* 0.3% (n=7) *Aedes africanus* 0.9% (n=17), *Culex eretmapodites* 0.5% (n=10) and *Aedes natalensis* 0.1% (n=2) respectively (Fig. 8). In addition, the greatest diversity was in the genus *Aedes* that recorded 3 species while the others species recorded 2 species each.

Table 3: Different mosquito species identified and their abundance at each sampling site in Kyela district, Tanzania

Species	**Kyela town**	**Ipinda**	**Kajunj.**	**Matema**	**Njisi**	**Total**
Ae. aegypti	120	100	100	100	60	**480**
Ae. natalensis	0	0	2	0	0	**2**
Ae. africanus	0	17	0	0	0	**17**
Cx. quinquefasciatus	280	20	200	0	240	**740**
Cx. eretmapodites	0	10	0	0	0	**10**
Mn. uniforminus	80	120	40	0	40	**280**
Mn. africanus	11	10	0	0	0	**21**
An. gambaie ssp	107	15	47	79	25	**273**
An. funestus	3	1	2	1	0	**7**
Total	**601**	**293**	**391**	**180**	**365**	**1830**

Table 4: Mosquito distribution by genus from each collection site in Kyela district, Tanzania

Site	Geographical coordinates	*Aedes*	*Culex*	*Mansonia*	*Anopheles*	Total
		N(%)	N(%)	N(%)	N(%)	
Kyela	S 09˚ 36’’ 270’’ E 033˚ 52’ 111’’	120 (24.4)	280 (57.0)	91 (18.5)	110 (39.2)	**601**
Ipinda	S:09˚ 29’ 246’’ E 033˚ 52’ 774’’	117 (42.2)	30 (10.8)	130 (46.9)	16 (5.7)	**277**
Kajunjumele	S 09˚ 35’ 809’’ E033˚ 55’ 551’’	102 (29.8)	200 (58.4)	40 (11.6)	49 (17.5)	**342**
Njisi	S 09˚ 35’ 481’’ E 033˚ 47’ 140’’	60 (17.6)	240 (70.5)	40 (11.7)	25 (8.9)	**340**
Matema	S 09˚ 29’ 767’’ E 034˚ 01’ 428’’	100 (20)	0 (0)	0 (0)	80 (28.7)	**100**
Total		**499 (27.2)**	**750 (41)**	**301 (16.5)**	**280 (15.3)**	**1830**

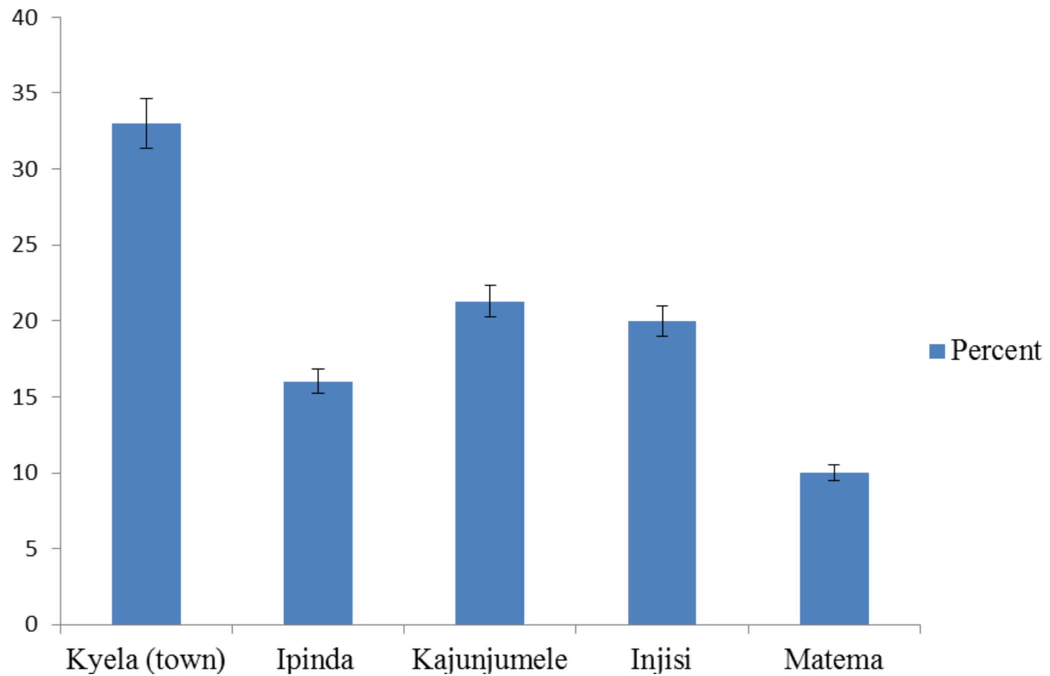

Figure 7: Abundance of the adult mosquito collected according to the sampling sites in Kyela district, Tanzania.

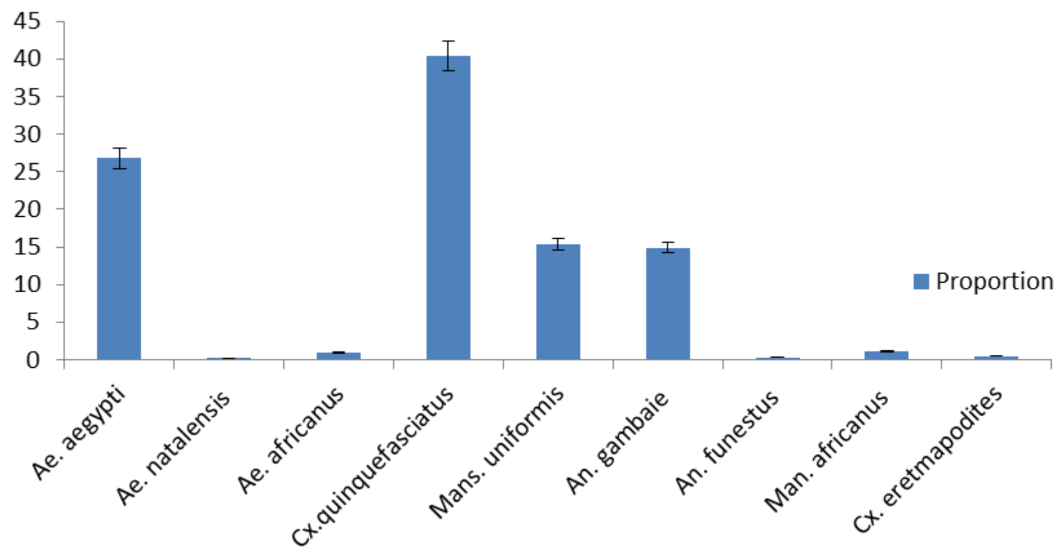

Figure 8: Different mosquito species collected and identified morphologically in the selected sites in Kyela district, Tanzania. Where is Anopheles???????

4.3 Detection of arboviruses specific genus in mosquitoes by RT-PCR

For the screening of arboviruses, only *Aedes* mosquitoes were used and pooled into 24 (20 mosquito in each pool) including 23 and 1 pools of *Aedes aegypti* and *Aedes africanus* respectively were stored in freezer at -20˚C. Specific arbovirus genus (*Bunyavirus*, *Alphavirus* and *Flavivirus)* were screened from them. From 24 *Aedes* pools screened, arboviruses were detected in 9 (37.5%) including *Alphavirus* (8 pools) and *Flavivirus* (1 pool). No sample has been positive for *Bunyavirus*. The positive mosquito samples (pools 2, 3, 4, 6, 7, 8, 9and 10) were then further tested using primers targeting conserved genes in the specific virus belonging to the concerned genus (Fig.9 and 11).

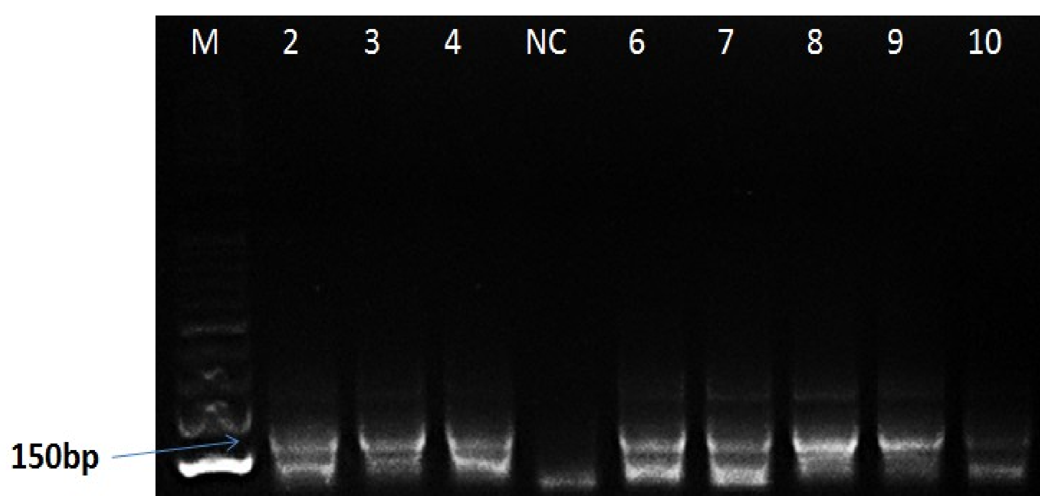

Figure 9: Visualization of RT-PCR product of the *Alphavirus* NSP4 gene.
M: DNA Ladder/Marker, NC: Negative control. Line 2-10: samples. The expected size of amplicon was 150 bp. Samples 2, 3, 4, 6, 7, 8 and 9 were positive, while sample 10 was weakly positive.

4.5 Arbovirus distribution and virus-specific infection status

From a total of 8 *Aedes* mosquito pools tested positive for *Alphavirus* genus, Chikungunya virus was identified into pools by RT-PCR, giving an infection rate of 75% (n=6). The highest number of Chikungunya infection was detected in mosquitoes sampled in Kyela town 66.6% (n= 4) followed by Kanjunjumele 16.6% (n=1) and Njisi 16.6% (n=1). No infection was found in Matema and Ipinda. (Fig.10). the statistic test showed that positivity rates were not significantly different among the different villages (P >0.05). However, the higher rates of infection were found in the more geographically central study sites, areas surrounding rice plantations.

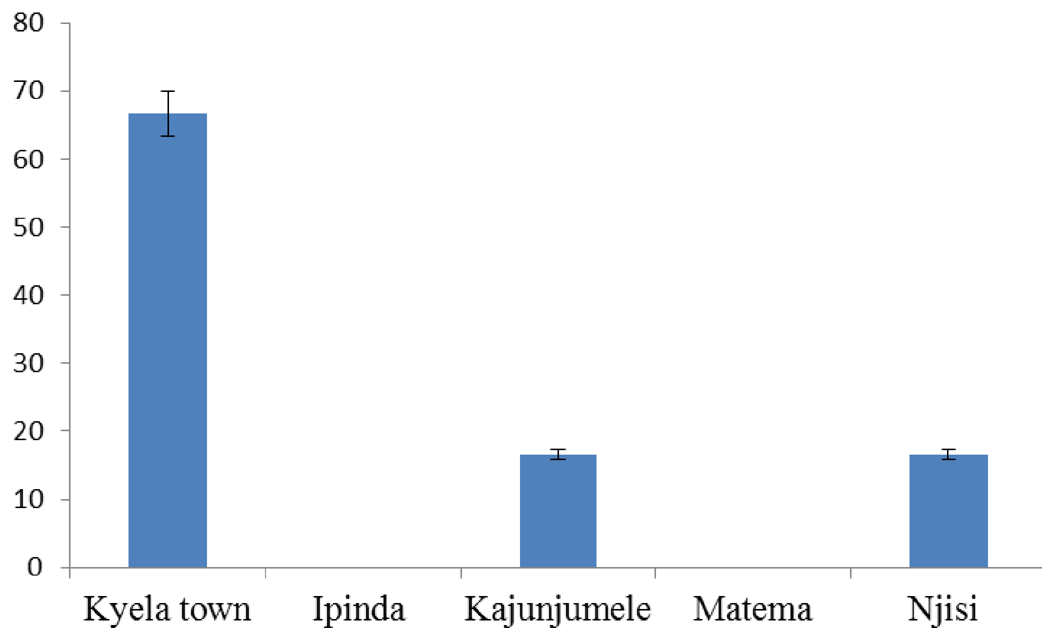

Figure 10: Village-specific positivity rates (%) for each of the five sampling villages

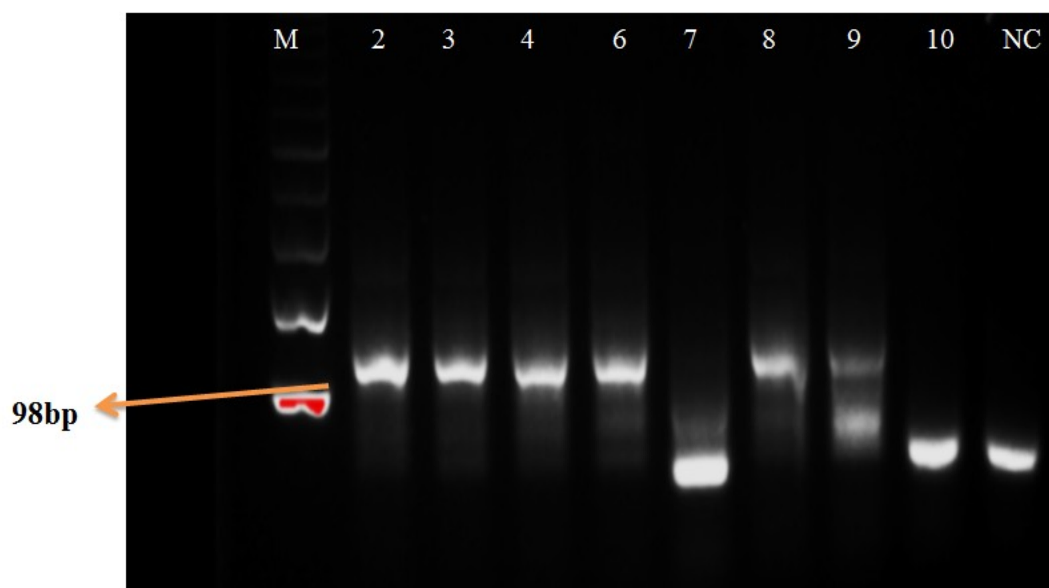

Figure 11: Visualization of RT-PCR products 5'NTR for Chikungunya virus. The expected PCR product size was 98 bp. M: DNA marker. Lines 2-10: Mosquito pools, NC: Negative control. Pools 2, 3, 4, 6, 8 and 9 were positive, while pool 7 and 10 were negative.

Table 5: Number of arboviruses identified in *Aedes* mosquitoes using RT-PCR

Sites	Number of pools	FlaV	BunyaV	AlphV	DFV	RVF	CHIKV	YFV
Kyela town	11	1*	0	6	0	0	4	0
Kajunjumele	6	0	0	1	0	0	1	0
Ipinda	3	0	0	0	0	0	0	0
Matema	2	0	0	0	0	0	0	0
Njisi	2	0	0	1	0	0	1	0
Total	**24**	**1***	**0**	**8**	**0**	**0**	**6**	**0**

***:** Weakly positive sample

CHAPTER FIVE

5.0 DISCUSSION

A geographical assessment of arbovirus vectors and their role in virus transmission is a fundamental aspect for the determination of high risk areas where emergence and circulation of arthropod-borne viral diseases might occur. The present study aimed to investigate mosquito abundance and their competence in arbovirus transmission in Kyela district. To our knowledge, this is the first study to be conducted in the above mentioned district on this topic. The importance of mosquitoes in arbovirus disease transmission and maintenance in Kyela distict located in South-Western Tanzania cannot be overemphasized. Thus, from April to May 2015, a total of 1830 adult mosquitoes were collected using mosquito Magnet traps, CDC light and HLC in five different villages in Kyela and were identified as belonging to nine species in four genera. The results obtained show that mosquitoes present in Kyela district belong mostly to *Culex*, *Aedes*, *Anophele*s and *Mansonia* genera representing tree species of *Aedes*, two of *Culex,* two *Anopheles* and two *Mansonia*.

The most abundant species identified was *Cx. Quiquenfiasciatus* 40.4% (n=740), followed by *Ae. Aegypti* 26.8% (n=480). Mosquito abundance differed dramatically among the ecologically distinct villages where the mosquitoes were collected. The abundance of these primary vectors and floodwater *Aedes* in the semi-arid district may be attributed to the nature of the terrain, soil types and vegetation cover, and rainfall which may influence availability of favorable vector breeding and resting

grounds (Lutomiah *et al*., 2013; Sang *et al*., 2008). The largest number of mosquitoes was collected from the central areas such as Kyela town 33% (n=601)) and Kajunjumele 21.3% (n= 391). This could actually be explained by the reason that, cattle owners in these areas have the habit of tethering their animals on the doorsteps of their houses at night for fear of theft, providing reservoir and potential attractant animals of several mosquito species particularly *Culex* and *Aedes* mosquitos (Matthew *et al*., 2008). In addition, irrigation system, uncontrolled growth in the central areas of Kyela, frequent water logging using for wetland rice cultivation increase highly the level of pollution. These observations are supported by studies from Cameroun (Hougard *et al*., 1993) and Kenya (Nguku *et al*., 2010) proving that the strong presence of *Cx. quinquefasciatus* can be considered as a biological marker of urbanization and most arboviruses vectors are found where soils retain water.

Aedes aegypti the principal vector of CHIK, DEN, RVF and YF viruses was predominated in the Kyela town where there is high range of temperature and uncontrolled level of population growth. This is due to the anthropophilic behavior of *Ae. Aegypti* (Harrington *et al*. 2001) feeding where there is high level of human activity, mismanagement of container after use, presence of old tyres attributing to lack of environmental hygiene. This is confirmed by the report from Pakistan showing that tyres trade had contributed to the reinvading of *Ae. Aegypti* major vector of emerging disease such as dengue in the area where it was already eradicated (Rasheed, 2012). With the evidence that East African *Ae. aegypti* are among the most competent vectors of YFin the world (Tabachnick *et al.,* 1985) also

means that the Kyela district is consistently at higher risk of YF transmission. The abundance of *Mn. uniformis* and *Mn. africana* in Ipinda and Kyela town characterized by flood plains is an indication that these species are adapted to the large swampy areas around rice cultivation where irrigation system is used. This is supported by study showing that these mosquito species are known to breed around flooded areas or around the edges of water bodies containing emergent vegetation, and to feed predominantly on livestock (Sang *et al*., 2010). A study from Kenya reported that Rift Valley fever virus was isolated from collections of these species from Baringo district during the 2006-2007 outbreaks (Sang *et al*., 2010); it is likely that these two villages are at high risk for Rift Valley fever virus transmission.

Distinct arboviruses were detected in *Aedes* mosquito pools collected in Kyela by RT-PCR. From 24 pools of *Aedes* species, arboviruses were detected in 9 including *Alphavirus* (8) and *Flavivirus* (1) genus; no sample tested positive for *Bunyavirus*. These results correlate with the recent studies carried out in the same district reporting high seroprevalence of *Alphaviruses* and *Flaviviruses* in human serum collected particularly in Kyela and Kajunjumele (Weller *et al*., 2014; Heinrich *et al*., 2012). This suggests that *Aedes* mosquitoes may have played an important role in the transmission of arbovirus in Kyela district.

The infectivity rates differed according to the different sampling sites. More infected pools of arboviruses were reported from Kyela town, Njisi and Kajunjumele; these three regions are classified as semi-arid, surrounding flood plains of Lake Malawi and are inhabited by communities whose economy mainly depend on paddy

cultivation. These conditions provide an ideal habitat for different *Aedes* species considered as major vectors for most arboviruses. These finding are corroborated by previous studies suggesting that most of arboviruses primarily affect inhabitants of the dry lands of the Rift Valley regions and the outlying semi-arid and arid grazing lands (Anyamba *et al*., 2001). In addition, despite the differences in geographic locations, arbovirus infectivity rate may be explained by the anthrophilic behavior of *Aedes* mosquitoes. Heinrich *et al*., (2012) showed that Chikungunya IgG was more evenly distributed in the two Kyela sub- sites and was also common in other sites nearby watercourse. It is estimated that apart from the trans-ovarial transmission which is common for most vectors, *Aedes* mosquitoes collected in these areas may feed on infected people and then become infected.

Amplification with specific primers of distinct viruses from suspected positive arbovirus genera showed the presence of Chikungunya virus. However, no sample was found to be positive for Dengue fever, Rift valley fever or yellow fever virus. Studies carried out in New California (Dupont *et al*., 2012) and in Argentina (Domingo *et al*., 2005) confirm the implication of *Aedes aegypti* in Chikungunya and Dengue transmission during epidemics. *Aedes spp* were showed also to be responsible for the 2004-2006 Chikungunya outbreak in the Indian Ocean Islands (Sang *et al*., 2008), where 75% of the population was affected (Sergon *et al*., 2008). While *Ae. aegypti* is usually responsible for urban yellow fever outbreaks, the first reported outbreak in 1992-1993 in the Rift Valley Province of Kenya was sylvatic in nature and was associated with *Aedes (Stegomyia) africanus.* Indeed, detection of infection of arboviruses in this area suggests that *Ae. spp* have the potential to

transmit Chikungunya and Dengue virus that they may play a major role during epizootics/epidemics. The low positivity obtained for genera-specific could be due to the mosquito preservation methods used as well as the time between sample collection and laboratory analysis. *Aedes* mosquito pools used in this investigation was frozen at – 20 ˚C and RNA extraction was done 4 months after collection. These situations may increase the chance for RNA degradation as cold storage availability allows samples to be stored not longer than a week after field collection (Sikulu *et al*., 2011). The most recommended preservation method for RNA extraction is RNAlater as well as Liquid nitrogen which is a nontoxic tissue storage reagent that rapidly permeates tissues to stabilize and protect cellular RNA. No pool was positive for Dengue fever, Rift valley fever and Yellow fever virus and this could be ascribed to absence of outbreaks reported for these diseases in the region suggesting that mosquitoes could be feeding on uninfected people.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The present study carried out during April and May 2015, period characterized by heavy rainfall in Kyela district located in South-Western part of Tanzania has shown the presence of different vectors (*Aedes*, *Culex*, *Mansonia* and *Anopheles ssp*) and that, vector species composition and abundance was different for each of the sampling sites. Using RT-PCR, arboviruses in the genera of *Alphavirus* particularly Chikungunya Virus were identified in *Aedes* mosquito pools showing an evidence of active circulation of these viruses in the study area. The findings of this study suggest that people from Kyela, especially in certain geographic locations, are highly likely to be exposed to arboviruses over the course of their lifetime. We conclude that, various and prevalent arboviruses of public health importance are carried by mosquitoes circulating in Kyela district and the environment is playing a role for their emergence.

6.2. Recommendations.

Based on the finding of the present study, the following are recommended:

(i) Further study on mosquito diversity on a large scale specially by collecting larvae are needed for good understanding of the entomological features to predicted infection risk for human as well as livestock,

(ii) Molecular characterization of Chikungunya virus for understanding distinct genotypes circulating in the area,

(iii) The remaining mosquito species which were not analyzed during this investigation underscores the need for further molecular studies to determine their competence for arbovirus transmission in Kyela,

(iv) Implementation of mosquito based arbovirus surveillance is therefore vital as part of an early warning systems that could provide information necessary for a rapid response plan against emerging arboviruses or emerging vector/virus associations of public health importance.

(v) In depth, genetic characterization of viruses is needed for recommendation of the national risk management and control of arboviruses infection in the studied areas and potential in contact regions.

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APPENDICES

Appendix 1: Mosquito collection form

SOKOINE UNIVERSITY OF AGRICULTURE						
FACULTY OF VETERINARY MEDICINE						
Adult Mosquitoes Collected by CDC-Light Trap/Mosquito Magnet						
istrict________________	Village:____________________________________					
ID__________________	Trap number________					
Coordinates____						
Mosquitoes Collected						
I. Mosquito sorting						
Genus	**Female**			**Males**	**Total**	
II. Mosquito identification to species						
Mosquito Species	Physiological status				Males	Total
	Unfed	Fed	Semi gravid	Gravid		
Notes (Operational constraints etc)						

Appendix 2: Purification of viral RNA

Five hundred and sixty microliter of prepared Buffer AVL containing carrier RNA was added into a 1.5 ml microcentrifuge tube and 140 µl mosquito supernatant, was added to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 sec; incubate at room temperature (15–25°C) for 10 min. The tube was then briefly centrifuged to remove drops from the inside of the lid. Five hundred and sixty microliter of ethanol (96–100%) was added to the sample, and mixed by pulse-vortexing for 15 s. After mixing, the tube was briefly centrifuged to remove drops from inside the lid. Carefully 630 µl of the solution from step 5 was added to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim, the cap was closed , and we centrifuged at 6000 x g (8000 rpm) for 1 min. Then we placed the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate. We carefully opened the QIAamp Mini column, and repeat step the above step. If the sample volume was greater than 140 µl, we have to repeat this step until all of the lysate has been loaded onto the spin column.

The bound nucleic acid was washed by adding to the QIAamp Mini column, 500 µl of Buffer AW1 and centrifuging at 6000 x g (8000 rpm) for 1 min. The second wash has been done bt adding 500 µl of buffer AW2 and then centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 min. The collection tube with the filtrate was discarded and replaced with a clean 1.5 ml micro centrifuge tube. RNA was eluted by adding 60 µl of elution buffer equilibrated to room temperature to the column. Viral RNA was stored at 20°C until amplification.