ARBOVIRUS INFECTIONS IN AEDES MOSQUITOES AT THE INTERFACE OF HUMAN-LIVESTOCK-WILDLIFE ECOSYSTEM IN KILOSA DISTRICT, TANZANIA

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

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ABSTRACT

Arboviruses refer to a group of viruses, which are transmitted by arthropods including mosquitoes and ticks. The objective of this study was to determine mosquito transmission potential of arboviruses at the interface of human-livestock-wildlife ecosystem in Kilosa district, Tanzania. Adult mosquitoes were collected using Mosquito Magnet® Liberty Plus traps. Reverse transcription polymerase chain reaction assay was performed on pooled adult Aedes mosquitoes to detect the presence of Dengue virus (DENV), Chikungunya virus (CHIKV), Rift Valley fever virus (RVFV) and Yellow fever virus (YFV). A total of 1340 mosquitoes belonging to four genera (Aedes, Anopheles, Culex and Mansonia) and 6 species were collected. Culex accounted for the largest (48.06%; n= 644) proportion of the mosquitoes while Anopheles for the lowest proportion (2.54%; n=34). Of the total mosquitoes collected, *Aedes aegypti* accounted for the majority of mosquito species (46%; n=613), followed by *Culex quinquefasciatus* (44.1%; n=591). Of the 36 *Ae. aegypti* pools tested for arbovirus 10 (28%) pools were positive. Dengue virus was detected in 3% (1/ 36) pools and CHIKV in 25% (9/ 36) pools. One pool that was positive for DENV also tested positive for CHIKV indicating the possibility of co-infection whereby, individuals may become infected by more than one arbovirus at a time thus risk of co-transmission to human and livestock. Nucleotide sequencing of polymerase chain reaction (PCR) products of the structural polyprotein region of DENV produced 511 bp fragment. Basic Local Alignment Search tool for nucleotides (BLASTn) and phylogenetic analysis showed that the (DENV3/TAN/Mikumi/2020 strain) (Accession number MW133786) obtained from this study clustered with DENV-3 strains reported in China and Kenya. This information is important as it gives knowledge on areas at high risk for arboviral disease outbreaks. The findings indicate that the presence of various mosquito vectors and detection of

arboviruses in wild-caught Aedes mosquitoes leave the population of Kilosa district at a higher risk of transmission of DENV and CHIKV.

DECLARATION

I, Ines Sebastian Machelle, do hereby declare to the Senate of the 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.

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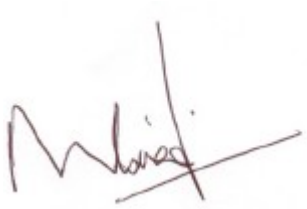

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Dr. Leonard E.G. Mboera Date

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DEDICATION

This work is dedicated to my parents Mr and Mrs Sebastian Machelle, my siblings; Irene Machelle, Cassian Machelle, Chrisostom Machelle and Curthbert Machelle and to all stakeholders in mosquito-borne viral diseases research and control. It is my hope that these findings will help each one of us in real life. This was a great motivation for me to undertake and complete this work.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

µL	microliter
BLASTn	Basic Local Alignment Search Tool Nucleotide
bp	base pairs
CDC	Centers for Disease Control and Prevention
CHIKV	Chikungunya virus
DENV	Dengue Virus
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
GPS	global positioning system
M	molecular marker
MBV	mosquito-borne virus
MEGA	Molecular Evolutionary Genetics Analysis
MRCC	Medical Research Coordinating Committee
NSm	non-structural protein
ORF	open reading frame
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
RVFV	Rift Valley fever virus
SUA	Sokoine University of Agriculture
TAE	Tris acetate EDTA buffer
URT	United Republic of Tanzania
UV	Ultraviolet
WHO	World Health Organization
YFV	Yellow fever virus

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Mosquitoes are vectors of many disease agents such as protozoa, viruses and worms that impact both human and animal health around the world (Bolling *et al*., 2015). The increased frequency of emerging and re-emerging infectious diseases is of concern worldwide. A large number of emerging infectious diseases are caused by arthropod-borne viruses (arboviruses) which are maintained in a sylvatic cycle between the vectors and vertebrate wildlife species, with spillover to humans in areas where there is human and wildlife population interface (Crabtree *et al.*, 2013).

Mosquitoes account for most of the arboviral infections globally. Mosquitoes feed on a variety of vertebrate hosts including wildlife that may represent unknown pathogen reservoirs, not only of known diseases but also of emerging infectious diseases (Daszak *et al.*, 2001). Sub-Saharan Africa has experienced several mosquito-borne viral diseases including Rift Valley fever, Chikungunya, Yellow fever, West Nile, Dengue and Zika that cause significant public health and socio-economic burden (Weaver and Reisen, 2010). Arboviruses comprise over 500 viruses mainly in three families *Togaviridae*, *Flaviviridae* and *Bunyaviridae* united by their arthropod vectors, namely mosquitoes, ticks and sandflies (Gan and Leo, 2014).

Arboviruses in nature are maintained through a complex cycle that involves arthropods as vectors which are involved in transmission of virus and vertebrate hosts that act as reservoirs of the virus. Humans in this cycle are usually dead-end hosts though they act as amplifying hosts for some arboviruses (Crabtree *et al.*, 2013). Vector surveillance is

critical for monitoring and predicting outbreaks as well as designing appropriate interventions of arboviral diseases (Crabtree *et al.,* 2013).

1.2 Problem Statement and Justification

Increasing prevalence of arbovirus infections is being evidenced in different places where studies have been conducted (Gan and Leo, 2014). Mosquitoes play a significant role in circulating these viruses between human, livestock and wildlife. Sylvatic circulations of virus with spill over to human/livestock involve direct transmission to human by primary enzootic vectors. Emerging infectious disease events are mostly attributed to zoonoses originating in wildlife with spillover to humans and are frequently caused by arboviruses (Crabtree *et al.,* 2013). Transmission occurs at an interface then spillover occurs from the enzootic cycle when human enter zoonotic foci and/or enzootic amplification increases circulation (Crabtree *et al.,* 2013). The aim of this study was therefore to fill the gap and establish baseline information on arbovirus disease transmission dynamics at human-wildlife-livestock interface where many mosquito-transmitted pathogens are endemic.

1.3 Objectives

1.3.1 General objective

The general objective was to determine mosquito transmission potential of arboviruses at the interface of human-livestock-wildlife ecosystem in Kilosa District, Tanzania.

1.3.2 Specific objectives

The specific objectives were:

i. To determine Aedes mosquito abundance and distribution at the interface of human-livestock-wildlife ecosystem in Kilosa District,

ii. To determine the circulating arboviruses in mosquitoes at the interface of human-livestock-wildlife ecosystem in Kilosa District, and
iii. To perform sequencing and phylogenetic analysis reconstruction of dengue virus at the interface of human-livestock-wildlife ecosystem in Kilosa District.

1.4 Research Questions

i. What are the transmission pathways of arbovirus between human, livestock and wildlife?
ii. What arboviruses are circulating in mosquitoes at the interface of human-livestock-wildlife ecosystem in Kilosa District?

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Mosquitoes as Vectors

Arboviruses are a group of viruses that are transmitted by arthropods including mosquitoes and ticks, causing a constant threat to human and animal health. Over 300 species of mosquitoes notably Aedes, Mansonia, Anopheles and Culex have been reported to transmit viruses (Braack *et al*., 2018). The Aedes mosquitoes particularly have been reported to harbour important pathogens such as RVFV, CHIKV, YFV and DENV (Braack *et al*., 2018).

2.1.1 Virus replication and distribution upon infection in mosquito

Transmission of arboviruses is naturally maintained by ability of viruses to infect mosquitoes, with the spread and replication in salivary gland (Gubler, 2001). Not all mosquitoes get infected after getting infectious blood meal, and not all infected mosquitoes can transmit the virus. For mosquito to be able to transmit virus, the virus must replicate in salivary glands and disseminate beyond midgut. Incubation period between taking viraemic 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).

2.1.2 Arbovirus status in Tanzania

Several arbovirus epidemics have been reported in different parts of Tanzania. Recent Dengue outbreaks was reported to occur in 2019 in Dar es Salaam, Tanga and Morogoro whereby Dar es Salaam has been the epicenter of the outbreak and past outbreaks

(Okada *et al*., 2019). Dengue virus was reported in 2010 among returning travellers in Europe, particularly patients with a travel history to Zanzibar, Tanzania (Gautret *et al*., 2010). Tanzania experienced the worst Dengue fever outbreak that has claimed lives in May 2014. These outbreaks were confirmed in several regions on the mainland, and other regions in Zanzibar (WHO, 2014). During the outbreak, mosquitoes collected particularly *Aedes aegypti* were reported to harbour DENV (Mboera *et al*., 2016).

On 25 June 2018, the Tanzanian Ministry of Health reported an outbreak of Chikungunya in Rombo in Kilimanjaro region involving four individuals travelling from Mombasa, Kenya. Before this incidence, in January 2018, six cases of Chikungunya virus were also diagnosed among another group of travellers from Mombasa (https:www.thecitizen. co.tz/ News/;https://www.dw.com/sw/). Chikungunya virus have also been reported in children in Kilosa (Chipwaza *et al.,* 2014) in Morogoro region. Evidence of Chikungunya virus infection among febrile patients seeking healthcare in selected districts of Tanzania; Sengerema, Kyela, Kirombero and Karagwe was reported by Kinimi *et al.* (2018). In addition, prevalence of Dengue and Chikungunya virus infections in north-eastern Tanzania has been reported by Kajeguka *et al.* (2016) in Hai, Babati, Moshi, Tanga and Handeni. Also, an acute CHIKV infection in a clinical study in Northern Tanzania confirmed by PCR has been reported by Crump *et al.* (2013). These studies and many others point to the presence of the virus affecting both human and livestock population suggesting that these viruses have become established.

Rift Valley Fever virus was first reported in Tanzania in 1930. Several studies have demonstrated the presence of RVFV antibodies in animals and human in some regions of Tanzania including Dodoma, Manyara, Morogoro, Dar es Salaam, Iringa, Mwanza, Singida and Tanga regions (Nderitu *et al*., 2011), also among inhabitants of Kilombero

(Sumaye *et al*., 2015). Kigoma district recorded RVFV for the first time in four years after the 2007 epizootic in Tanzania in livestock (Kifaro *et al*., 2014).

2.2 Arboviral Diseases

2.2.1 Rift Valley fever

Rift Valley fever (RVF) is a zoonotic disease caused by RVF virus with impact on animal and human health. RVF outbreaks have been known to occur every 10 years following heavy rainfall (Sindato *et al*., 2014). Incubation period ranges from one to three days followed by fever, haemorrhagic diarrhoea, recumbency and high rates of storm abortion in cattle, sheep and goats (CDC, 2013). Mortality in cattle, sheep and goats can reach 70% (Johnson *et al.,* 2012). The virus causes disease in humans ranging from influenza-like illness to haemorrhagic fever with hepatomegaly and encephalitis (Bouloy and Weber, 2010).

In domestic animals, RVFV is transmitted either through a bite by infected mosquitoes or direct contact with infected animal tissues, body fluids and fomites (Woods *et al*., 2002; Pepin *et al.,* 2010). In humans, various transmission routes have been reported and they vary in their contribution based on epidemic stages (Woods *et al*., 2002; Pepin *et al.,* 2010). The transmission through bites by RVFV infected mosquito is predominant during the first stages of outbreak while direct contact with infected animal tissues, blood, or other body fluids is predominant during disease amplification stage (Woods *et al*., 2002; Pepin *et al.,* 2010). RVFV persists in an environment through vertical transmission within mosquitoes and horizontal transmission between mosquitoes and animals. Different mosquito species are involved in transmission of RVFV including, *Ae. aegypti, Ae. vexans, Ae. simpsoni, Ae. pembaensis, Ae. ochraceus, Cx. quinquefasciatus, Cx. poicilipes, Cx. bitaeniorhynchus, Cx. univittatus, Anopheles squamosus* and *Mansonia uniformis* (Woods *et al*., 2002; Pepin *et al.,* 2010).

Rift Valley Fever virus is a member of the genus Phlebovirus of the family *Bunyaviridae.* It is a tripartite negative single stranded RNA genome with the diameter of approximately 80-120nm. The virus genome consists of three segments, a large segment (L) that encodes for an enzyme RNA dependent RNA polymerase which helps in virus transcription and replication, Medium segment (M) which encodes for non-structural protein (NSm) and structural protein where by NSm helps virus replication in cell culture, and also act as an ant apoptotic protein and structural protein helps the virus in receptor recognition, entry and budding. The last segment is a small segment (S) which is an ambience having positive and negative sense, whereby negative sense encodes for nucleo-capsid protein which binds the viral RNA, provide structural stability to the genome and helps in virus replication and assembly while positive sense encodes for non-structural protein NSs which serve as a virulent factor for the virus by inhibiting the host innate immune response (Pepin *et al*., 2010).

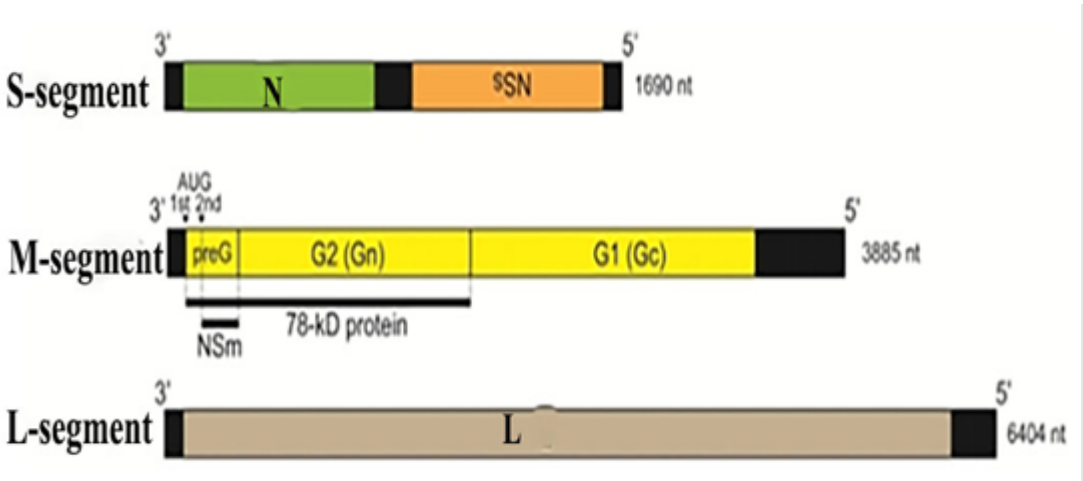

Figure 1: Genomic organization of RVFV genome

Source: Ikegami and Makino (2011)

2.2.2 Dengue fever

Dengue fever is a febrile illness caused by DENV, which infects almost 400 million persons worldwide every year (Okada *et al*., 2019). Dengue commonly manifest itself as a self-limiting flu that is characterized by a sudden onset of fever, retro-orbital headaches, myalgia, arthralgia, and leucopenia. There are now four antigenically distinct serotypes which are DENV 1-4 and although they share identical epidemiological features, they are genetically distinct (Holmes, 1998). Dengue virus is a member of the family *Flavividae* of genus Flavivirus. 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 (ORF) 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).

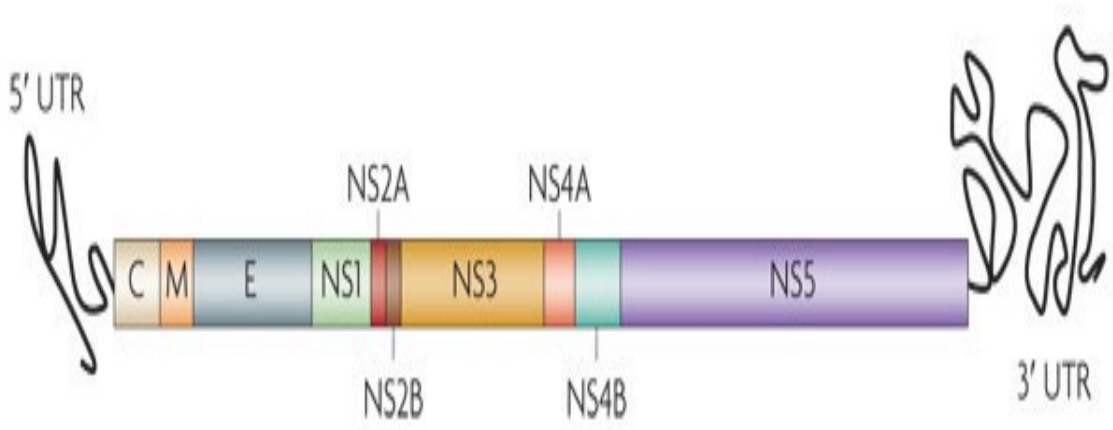

Figure 2: Flavivirus genome showing the non-segmented RNA strands.
Source: Guzman *et al*. (2010).

2.2.3 Yellow fever

Yellow fever is an acute disease caused by YFV, an arbovirus of the family *Flaviviridae*. Non-human primates such as monkeys are the main hosts of the virus, while humans become infected accidentally (Pinheiro *et al*., 2019). The main vector is *Ae. aegypti*. Yellow fever has an incubation period of 3 to 6 days and sudden onset of fever, myalgia, headache and nausea/vomiting. The disease ranges from asymptomatic to severe forms and the most serious forms occur in around 15% of those infected, with high lethality rates. These forms lead to renal, hepatic and neurological impairment and episodes of bleeding. Prevention is achieved by vaccination, which is safe and effective (immunogenicity at 90-98%) (Litvoc *et al*., 2018).

2.2.4 Chikungunya

Chikungunya virus infection has two stages, the acute illness and persistent arthropathy (chronic illness). Headache, fatigue, high fever within 4-7 days, polyarthralgia, backache, and digestive disorders at lower rate in acute disease. Symptoms in persistent arthropathy include fever, fatigue, rheumatoid arthritis, spondylarthopathy, headache, neuropathic pain syndrome and rashes (Win *et al.,* 2010).

Human serve as reservoir host for CHIKV during epidemics while during inter-epidemic period, monkey, rodents and birds are reservoirs (Rezza *et al.,* 2007). *Aedes aegypti* and *Ae. albopictus* are main vectors in transmission of CHIKV (Thiberville *et al.,* 2013).

Chikungunya virus is one of the most important Alphaviruses of human belonging to family *Togaviridae* that was first isolated in Tanzania in 1953 during an outbreak of human febrile illness accompanied by severe arthralgia and rash (Robinson, 1955). Alphavirus are enveloped with spherical virions, 60 to 70 nm in diameter. CHIKV genome is 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, E2 and E1 proteins (Fig. 3) (Strauss and Strauss, 1994). There are three CHIKV genotypes with different antigenic determinants including, Asian, West African and Indian Ocean (Eastern/ Central Africa) groups (Schuffenecker *et al.*, 2006).

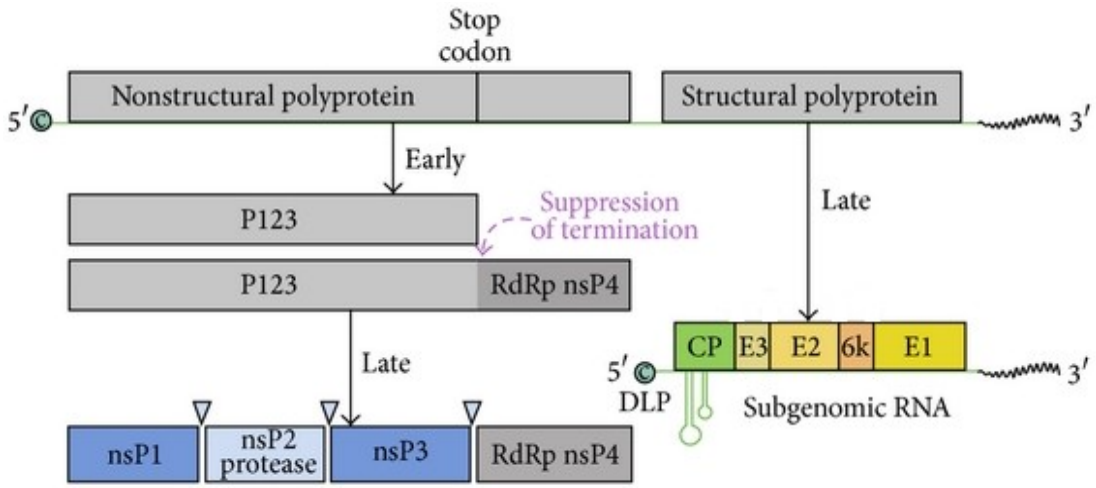

Figure 3: Alphavirus genome organization

Source: Hernandez *et al*. (2014).

The non-structural domain encodes four viral non-structural proteins (nsP1- nsP4), which are essential for replication and polyprotein processing (Fig. 3). In addition to copying the RNA genome, the non-structural proteins synthesize 26s sub-genomic mRNA which is capped and polyadenylated, producing five individual structural proteins which consist of the viral capsid (C), two envelope glycoproteins (E1 and E2) and two peptides (E3 and 6K) (Strauss and Strauss, 1994).

2.3 Principle of Reverse Transcription Polymerase Chain Reaction

In principle, viral RNA is extracted from mosquito sample, transcribed to cDNA in a reverse transcription (RT) reaction either in a separate reaction or in a one-step format and amplified by polymerase chain reaction (PCR). Depending on the purpose, the amplification targets include highly conserved regions of the DENV genome, such as the NS5 and 3' UTR, or areas with more variability, such as the C-preM and E-gene regions, Glycoprotein 2 gene for RVFV, E1 gene for CHIKV and NC gene for YFV. The products of the PCR will be sequenced to know the evolution of the virus.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This study was conducted in Kilosa district, Tanzania. Kilosa is one of seven districts of Morogoro region. It is situated between latitudes $5^0$55’ and $7^0$53’ South of Equator and longitudes $36^0$31’ and $37^0$30’ East of Greenwich meridian. The district covers 12 394 km^2 and is divided into 35 administrative wards and 118 registered villages. A total of 212 500 hectares of the district is covered by a game reserve and Mikumi National Park. The National Park alone covers an area of 3230 km^2. The district population is estimated to be 438 175. The study involved 3 villages namely Mikumi, Ihombwe and Lumango which are located on the periphery of the Mikumi National Park (Figure 4). All sampling points within the study area were recorded using global positioning system (GPS) and mapped using Geographical Information System.

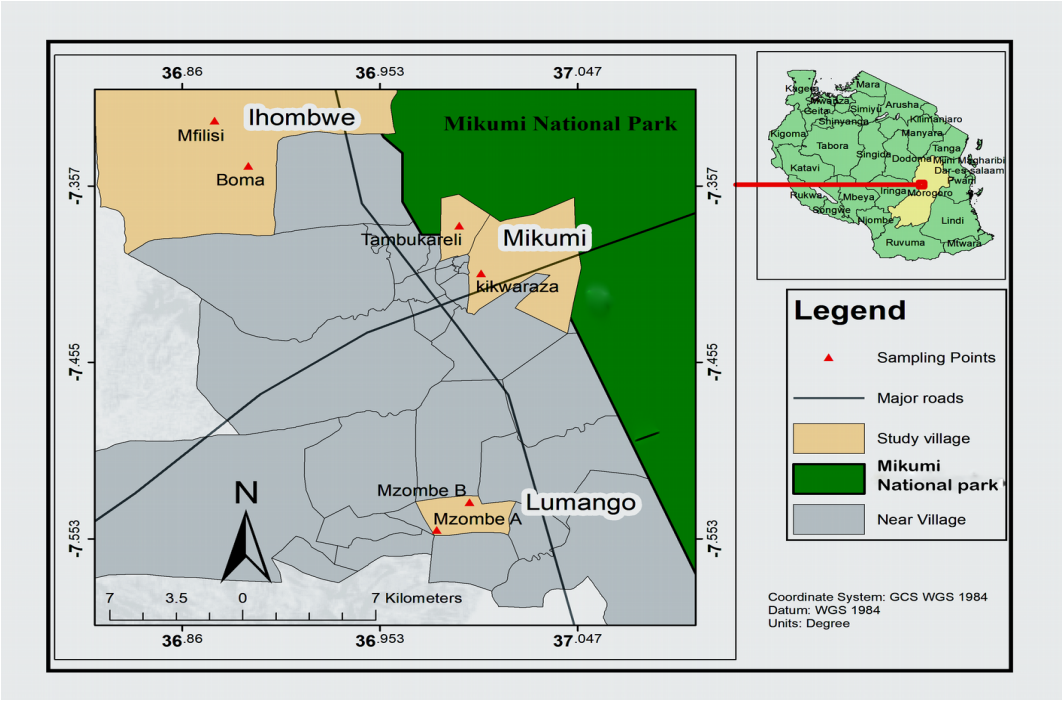

Figure 4: Map of Kilosa district showing villages and sampling points

3.2 Study Design and Sampling Technique

A cross-sectional entomological survey was carried out in three villages. Selection of villages was purposely done based on accessibility of the area. For each village 2 sites were selected for adult mosquito sample collection. Selection of sites for placement of mosquito traps was by systematic random sampling whereby all houses were arranged in an ordering scheme then the first house was randomly chosen. It involved a random start and then proceeded with skipping one house after the selection of every one house afterward. Mosquito collection was carried out between December 2019 and January 2020. Mosquito sampling forms were used to record mosquito and ecological data (Appendix 1) and all sampling sites were georeferenced using a hand-held Global Positioning System (GPS) device.

3.3 Data Collection Method

3.3.1 Adult mosquito collection

Mosquitoes were collected using Mosquito Magnet Liberty Plus traps (American Biophysics Corporation, Rhode Island, USA) baited with propane gas. In each village, traps were set to run for 24 hours from 0600 hours and then mosquitoes were collected each morning of the next day at 0600 hours for three consecutive days. Mosquitoes were placed in a paper cup for further preservation and identification.

Figure 5: Mosquito Magnet Liberty Plus traps used in mosquito sampling

3.3.2 Mosquito larvae and pupae sampling

Mosquito larvae and pupae were sampled from different locations using the standard dipping technique. In every site, all water-holding containers found were examined for the presence of larvae and/or pupae. The larvae were collected using large-mouth pipettes and a standard plastic dipper. The larvae/pupae were collected into a small bowl, sorted out and transferred into paper cups using Pasteur pipette. The samples were kept at room temperature for larvae and pupae to hatch.

3.3.3 Mosquito preservation and identification

Adult mosquitoes were immobilized in a freezer at -20 °C for 15 minutes. Mosquito specimens were sorted according to species using standard taxonomic and morphological identification keys described by Hopkins *et al.* (1952) under a stereomicroscope.

Mosquitoes were pooled into groups of 20 mosquitoes according to their sex, species, whether fed or unfed, and location and packed in cryovials. Mosquitoes were preserved in liquid nitrogen and transported to SACIDS Laboratory at Sokoine University of Agriculture in Morogoro before being preserved in an ultralow freezer at -80 °C for later screening of mosquito-borne virus. The adult female mosquitoes that emerged from larvae were also pooled.

3.4 Molecular Detection of Arboviruses

3.4.1 Pooling and homogenization of mosquitoes

Mosquitoes were pooled in which an average of 20 mosquitoes were pooled together into labelled 1.5 mL cryovial tubes according to their species and collection site. Thereafter, 500 µL of culture medium Dulbecco's Modified Eagles Medium (DMEM) was added into each of cryovial tube with pooled mosquitoes followed by grinding of mosquitoes by using micropestle to homogenise the mixture until homogeneous lysate was formed. Homogenized lysate was divided into two portions using Eppendorf tubes, one was to be used for RNA extraction and the other for virus isolation. Homogenized lysate was stored at -80 °C until further use.

3.4.2 Viral RNA extraction from mosquitoes

Total viral RNA was recovered from mosquitoes using QIAamp Viral RNA Mini extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions (Appendix 2). Briefly, samples were lysed using a lysis buffer followed by chemical protein precipitation using absolute ethanol. Protein precipitates were pelleted by centrifugation and the supernatant were passed through a silica column to trap the RNA based on charge differences. Column-bound RNA was washed with two different buffers and the column dried by high speed centrifugation. Afterwards, column-bound RNA was eluted with RNase free water. Extracted viral RNA was stored at -80 ºC until reverse transcription polymerase chain reaction (RT-PCR) was performed.

3.4.3 Arbovirus detection by reverse transcription polymerase chain reaction (RT-PCR)

The extracted viral RNA was used for one step RT-PCR amplification (AgPath-ID™ One-Step RT-PCR Kit, Thermo Scientific Cat. No. 4387391) using primers targeting specific arboviruses (Table 1). A total of 25 µL of master mix was prepared containing 12.5 µL of 2× RT-PCR buffer, 1 µL of 25× RT-PCR Enzyme Mix, 1 µL of both forward and reverse primer, 5 µL of RNA, 0.5 µL of 25 $MgCl_2$ and 4 µL of nuclease-free water up to 25 µL. Primers that target conserved genes in the specific viruses belonging to the genus in question such as CHIKV, RVFV, YFV and DENV (Table 1) were used to detect the presence of arbovirus specific genera. The following PCR cycling conditions were performed for DENV: Reverse transcription step at 48°C for 30 minutes, an initial denaturation step at 94°C for 2 minutes, followed by 35 cycles of denaturation at 94°C for 15 seconds, annealing temperatures at 55°C for 30 seconds each, elongation at 68°C for 1 minute and final extension at 68°C for 5 minutes.

Table 1: Primers used for the screening of arboviruses

Virus	Primer	Sequence (‘5 -> 3’)	Target gene	Genome position	Size (bp)	Reference
DENV	D1	TCAATATGCTGAAACGCGCGAGAAACCG	Structural polyprotein	134-161	511	Lanciotti *et al*., 1992
	D2	TTGCACCAACAGTCAATGTCTTCAGGTTC		616-644		
RVFV	RVF1	GACTACCAGTCAGCTCATTACC	Glycoprotein M gene	777-798	551	Ibrahim *et al*., 1997
	RVF2	TGTGAACAATAGGCATTGG		1309-1327		
YFV	CAG	CGAGTTGCTAGGCAATAAACACATTTGGA	Polyprotein	43-71	670	Onyango *et al.*, 2004
	YF7	AATGCTCCCTTTCCCAAATA		1293-1312		
CHIKV	CHIKV E1F	ACGCAATTGAGCGAAGCAC	E1	10294-10312	200	Thavara *et al*., 2009
	CHIKV E1R	CTGAAGACATTGGCCCCAC		10498-10480		

3.4.4 Gel electrophoresis and visualization of PCR products

PCR products were separated by electrophoresis, 1.5% of agarose gel, 0.5% of Tris acetic acid and 4 µL of GelRed (Phenix, Candler, USA) were used. Five µL of RNA sample, one µL of 6× loading dye and four µL of one thousand DNA marker (Promega, Fitchburg, CA) were loaded and run at 100 volts for 35min. The agarose gel was then visualized through ultraviolet fluorescence light by using gel documentation system (EZ Gel Doc, BioRad, USA).

3.5 Sequencing

PCR products (5 µL) obtained using universal Dengue virus primers D1 and D2 (Table 1) were denatured at 94°C for 3 minutes in order to remove unused primers and dNTPs, respectively. Sequencing of PCR products was done directly with universal Dengue virus primers D1 or D2 using the Big Dye Terminator Cycle Sequencing Kit Version 3.1 Genetic Analyser 3710 XL (Applied Biosystems, Foster City, CA).

3.6 Nucleotide Sequencing, Similarity Search and Determination of Phylogenetic Relationship of Dengue Virus

The forward and the reverse complement nucleotide sequences delimited by D1 forward and D2 reverse primers of structural polyprotein region PCR products of DENV were aligned to obtain a consensus nucleotide sequence. The nucleotide sequences of DENV structural polyprotein region was submitted to GenBank, and compared with other sequences using BLASTn (Altschul *et al*., 1990). Basic Local Alignment Search tool for nucleotides compares nucleotide sequences to sequence databases and calculates the statistical significance of matches and can be used to infer functional, and evolutionary relationships between sequences. A set of sequences representing DENV1-4 serotypes, together with those obtained from the present study, were used for phylogenetic analysis.

Sequences were aligned using ClustalW algorithm in BioEdit (Ibis Biosciences, Carlsbad, CA) and clustering pattern was determined by maximum likelihood method in MEGA 7® software (Tamura *et al*., 2011).

3.7 Data Analysis

Microsoft Office Excel 2019 program (Microsoft Corporation, Washington, USA) was used to organize and filter data. Further analysis to calculate proportion of mosquito composition and abundance was done by using R software version 3.5.3 (University of Auckland, Auckland, New Zealand).

3.8 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 (Ref. No: NIMR/HQ/R.8a/Vol.IX/3262). Owners of the premises where traps were set were requested for their permission before installation of mosquito traps in their house premises.

CHAPTER FOUR

4.0 RESULTS

4.1 Mosquito Abundance and Distribution

A total of 1340 mosquitoes were collected. Of these, 1255 mosquitoes were collected as adults and 85 as larvae and pupae. Most mosquitoes (43.7%; n=586) were collected in Lumango. Others were collected from Mikumi (39.48%; n = 529) and Ihombwe (16.79%; n = 225). Four mosquito genera (Aedes, Anopheles, Culex and Mansonia) were identified (Table 2). In this study, a total of six species were identified (Table 3). Among members of the genus Aedes collected were *Ae. aegypti* and *Ae. africanus*. The generic richness was predominant at Ihombwe, which had four genera with 6 different species. Lumango had 4 genera with five different species and Mikumi recorded three genera with 4 different species, respectively (Table 3) (Figure 6).

Among the 619 (46.19%) Aedes mosquitoes collected, *Ae. aegypti* accounted for 46.29% (n=613) while *Ae. africanus* accounted for 0.45% (n=6). Culex genus recorded the highest number of mosquitoes sampled (48.06%; n=644) (Table 2). The majority (44.64%; n=591) were *Cx quinquefasciatus* (Fig. 6). *Cx cinereus* accounted for only 3.96% (n=53) of Culex mosquitoes. All the Mansonia mosquitoes collected were identified as *M. uniformis*. *An. gambiae* accounted for all 2.54% of all the 34 Anopheles mosquitoes collected.

Table 2: Mosquito distribution (%) by genus by villages

Sampling village	Aedes N (%)	Culex N (%)	Mansonia N (%)	Anopheles N (%)	TOTAL N (%)
Mikumi	481 (77.71)	45 (7.08)	0 (0.00)	3 (8.82)	**529 (39.48)**
Ihombwe	66 (10.66)	152 (23.90)	6 (13.95)	1 (2.94)	**225 (16.79)**
Lumango	72 (11.63)	447 (69.04)	37 (86.05)	30 (88.24)	**586 (43.73)**
Total	**619 (46.19)**	**644 (48.06)**	**43 (3.21)**	**34 (2.54)**	**1340**

Table 3: Number (%) of mosquito species collected by village

Species	Mikumi	Ihombwe	Lumango	Total
Ae. aegypti	481 (90.93)	60 (26.67)	72 (12.63)	**613**
Ae. africanus	0(0)	6 (2.67)	0 (0)	**6**
Cx. quinquefasciatus	36 (6.81)	148 (65.78)	407 (71.4)	**591**
Cx. cinereus	9 (1.7)	4 (1.78)	40 (6.83)	**53**
Ma. uniformis	0 (0)	6 (2.67)	37 (6.49)	**43**
An. gambiae	3 (0.57)	1 (0.44)	30 (5.26)	**34**
Total	**529 (39.48)**	**225 (16.72)**	**586 (43.73)**	**1340**

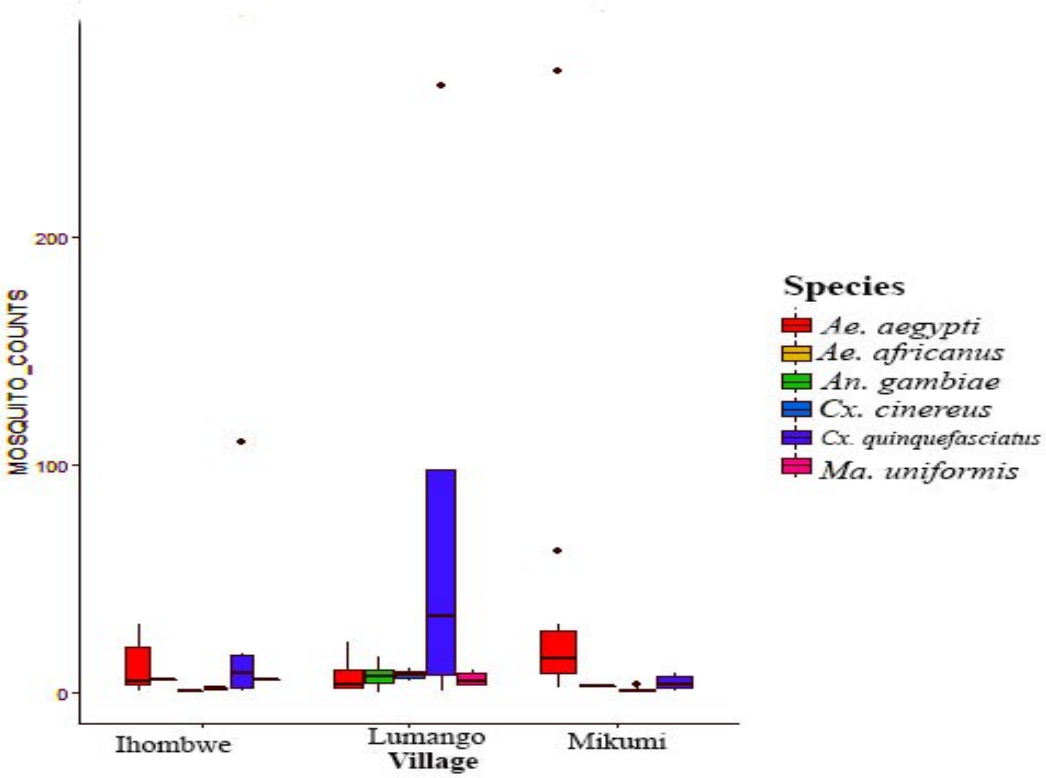

Figure 6: Distribution of mosquitoes species by village at Kilosa district

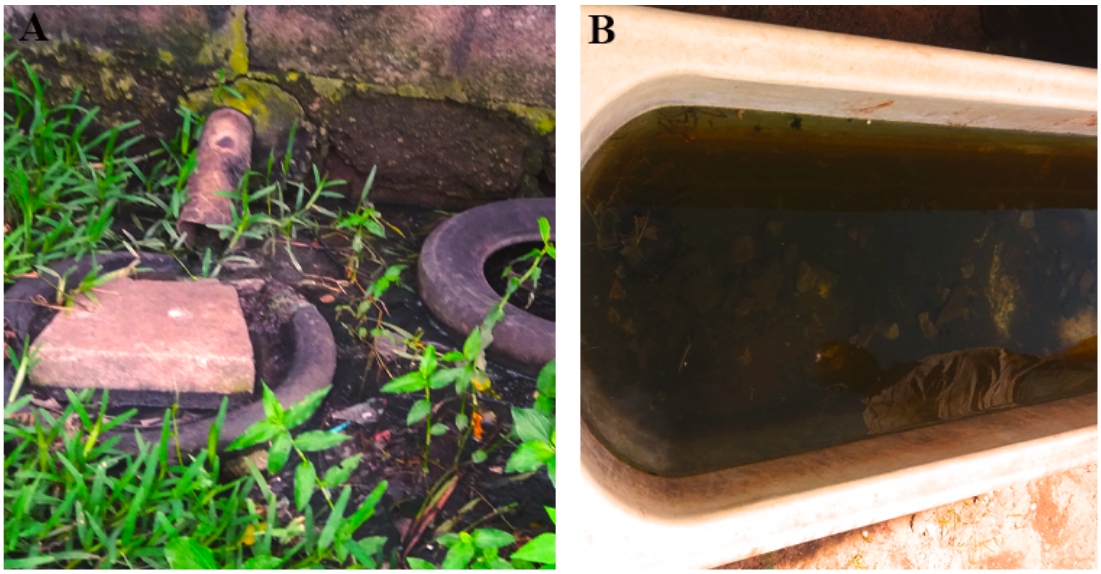

Figure 7: Habitats for larvae mosquito

Table 4: The habitats of adult mosquitoes and larvae/pupa in Kilosa district

Village	Type of habitat	Mosquito species
Adult mosquitoes		
Mikumi	Vegetation, Plain terrain, village	*Aedes aegypti, Culex quinquefasciatus, Culex cinereus* and *Anopheles gambiae*
Mikumi	Bush, hill, presence of animals	*Aedes aegypti, Culex quinquefasciatus, Culex cinereus*
Ihombwe	Plain terrain, village	*Aedes aegypti, Culex quinquefasciatus, Culex cinereus*
Ihombwe	Bush, valley terrain	*Aedes aegypti, Aedes africanus, Culex quinquefasciatus, Mansonia uniformis* and *Anopheles gambiae*
Lumango	Plain terrain, village, vegetation, presence of animals	*Aedes aegypti, Culex quinquefasciatus* and *Mansonia uniformis*
Lumango	Village, hill, evergreen forest, presence of house	*Aedes aegypti, Aedes africanus, Culex quinquefasciatus, Mansonia uniformis* and *Anopheles gambiae*
Mosquito larvae/pupa		
Ihombwe	Water holding	*Aedes aegypti*
Lumango	Discarded tin containers, bottles and broken pots	*Aedes aegypti* and *Culex cinereus*

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; Ibrahim *et al*., 1997). The expected PCR products were 511, 200, 551 and 670 bp for DENV, CHIKV, RVFV and YFV, respectively. Out of 36 Aedes mosquito pools tested, infections with DENV was observed in one pool and CHIKV were observed in nine pools (Table 5). One pool was positive for DENV and CHIKV. However, no pool was positive for RVFV or YFV.

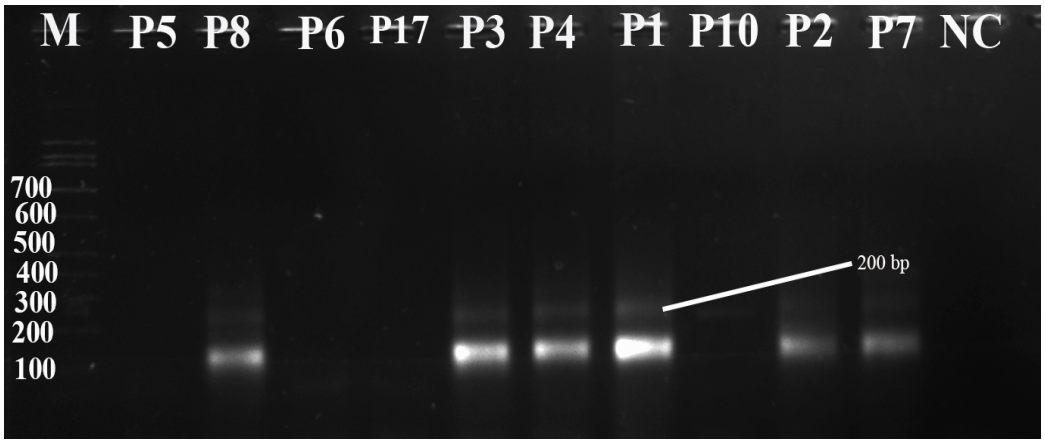

Figure 8: Detection of Chikungunya virus in mosquitoes

CHIKV E1F and CHIKV E1R primers were used to screen Chikungunya virus in mosquitoes using RT-PCR. The expected PCR product size is 200 bp. Lane 8, 3, 4, 1, 2 shows a suspected Chikungunya virus (CHIKV) and the rest shows negative. M- DNA marker, NC- negative control.

4.3 Dengue and Chikungunya Virus Infection Rate by Location

Of the 36 *Ae. aegypti* pools tested, arboviruses were detected in 10 (28%) pools. Dengue virus was detected in 3% (1/ 36) pools and Chikungunya virus in 25% (9/ 36) pools. DENV detected from Lumango village was 14.3% (1/7) while CHIKV accounted for 14.3% (1/7). CHIKV detected from Mikumi village was 25% (6/24), in Ihombwe village CHIKV infection rate was 20% (1/5). DENV were only detected in Lumango, but not Ihombwe and Mikumi. CHIKV were detected in Aedes mosquitoes in all three sampling locations, i.e. Mikumi, Ihombwe and Lumango.

Table 5: Arboviruses infection rate in Aedes mosquitoes

Location	Pool number	DENV	CHIKV	Infection rate per location
Mikumi	2	–	+	
Mikumi	3	–	+	
Mikumi	4	–	+	
Mikumi	5	–	+	
Mikumi	7	–	+	
Mikumi	1	–	+	25.00%
Ihombwe	10	–	+	
Ihombwe	8	–	+	20%
Lumango	12	+	+	14.3% DENV & CHIKV
Total		1	9	
Infection Rate		1/36 (2.8%)	9/36 (25%)	

4.4 Similarity of DENV RNA Sequence against GenBank

The obtained nucleotide sequence was submitted to GenBank. BLASTn of the DENV obtained from the present study showed high identity with a number of published DENV-3 sequences, of which these DENV sequences at Gen Bank had 100% identity. Tanzanian DENV showed highest identity with other DENV from China and Kenya. Evolutionary history was inferred using the Maximum likelihood method and analyses were conducted in MEGA 7.

4.5 Phylogenetic Analysis of DENV

A phylogenetic tree for the structural polyprotein region was constructed using the maximum likelihood method using DENV sequences retrieved from GenBank representing all the four DENV serotypes (Fig. 9). The tree revealed that the DENV sequence obtained from this study (DENV3/TAN/Mikumi/2020) (Accession number MW133786) clustered into serotype III and closely related to the 18XN10607 (Accession number MK894339.1) which is a DENV 3 isolated from China (Su *et al*., 2018) and (Accession number KX812511.1) (Konongoi *et a*l. 2016) which is DENV 3 strain isolated

from Kenya. Phylogeny was inferred following 1000 bootstrap replications as shown in Figure 9.

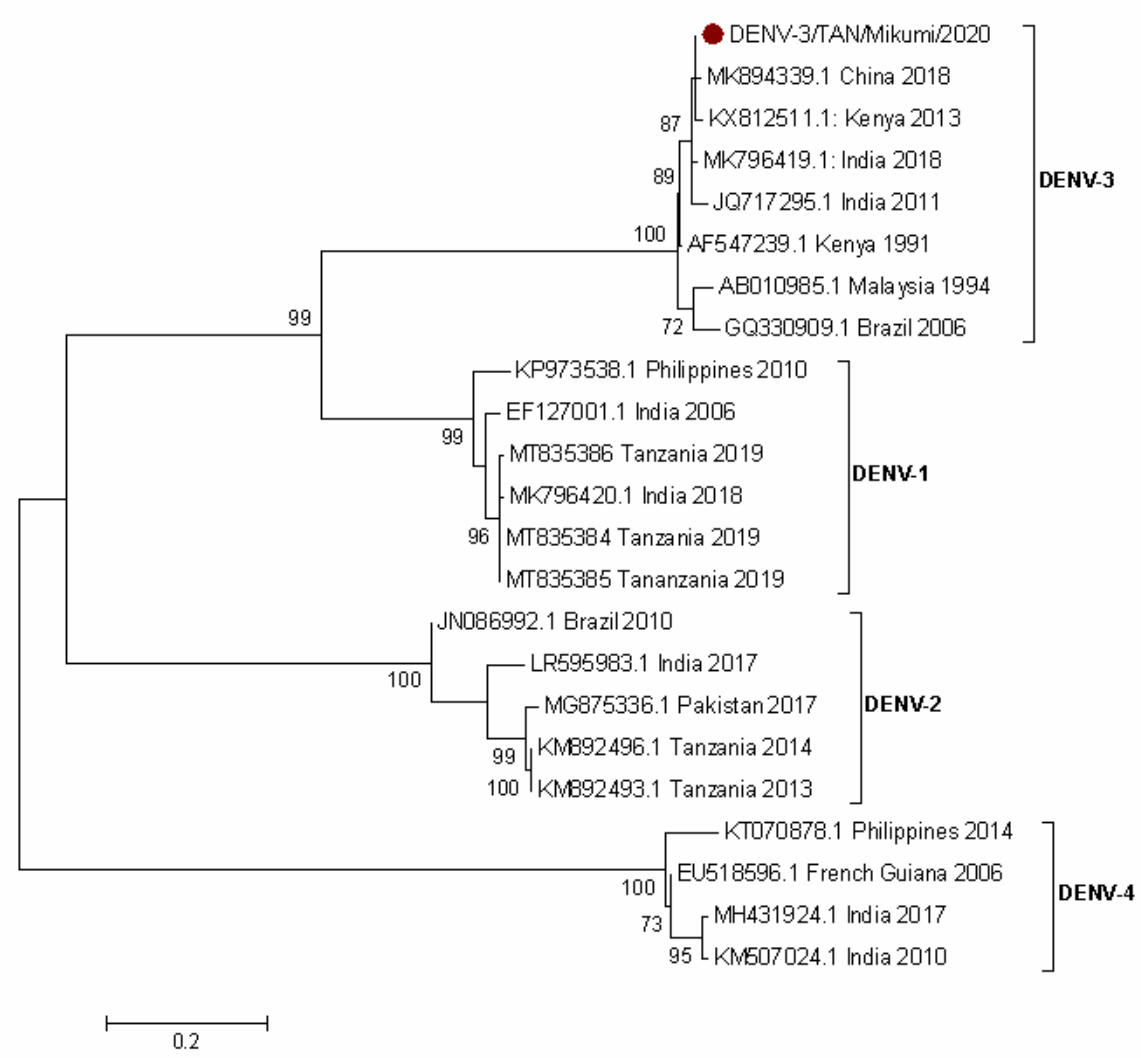

Figure 9: Phylogenetic tree of Dengue viruses based on the structural polyprotein region constructed using the maximum likelihood method in MEGA 7® software. The percentage of successful bootstrap replication is indicated at the nodes. On top is the isolated DENV-3 strains (DENV3/TAN/Mikumi/2020) indicated with a mark.

CHAPTER FIVE

5.0 DISCUSSION

In present study, Aedes mosquito abundance and distribution in selected sites at the interface of human-livestock-wildlife ecosystem in Kilosa was investigated. Culex accounted for the largest proportion while Anopheles for the lowest proportion of the mosquitoes collected. In a similar study in rural Ngorongoro district, Culex was found to be dominant (Mweya *et al.,* 2015). The higher relative abundance of Culex can be explained by the presence of favourable breeding sites in the sampled locations. *Ae. aegypti* was the most abundant species compared to all other species collected at the interface of human-livestock-wildlife ecosystem in Kilosa. The abundance of *Ae. aegypti* observed could be due to their breeding adaptation in vegetation and water holding containers (Mboera *et al*., 2016). Among all collected mosquito, one-third of Aedes mosquitoes were collected in areas covered with vegetation and discarded water holding containers which were found in garage and collection sites. Findings from this study showed the close proximity between *Ae. aegypti* with human-livestock and wildlife population due to their adaptation in their breeding site.

In the present study, CHIKV and DENV were detected in Aedes mosquitoes and their infection rate was determined. One pool that was positive for DENV also tested positive for CHIKV. Isolation of multiple arboviruses in the same pool of *Aedes species* indicates possibility of concomitant infections in the vectors. This is because mosquitoes can feed multiple times on more than one human host. Concomitant infections were reported in other studies by (Goertz *et al*., 2017; Coupanec *et al*., 2017 and Ruckert *et al*., 2017). This indicates the possibility of co-infection whereby, individuals may become infected by more than one arbovirus at a time. Thus, increasing the possibility for co- transmission from mosquito to human and livestock as well as reported by (Ngala *et al*., 2017).

However recent clinical data support an increase in the frequency of coinfection in human patients, raising the likelihood that mosquitoes could be exposed to multiple arboviruses during one feeding episode. Though the impact of coinfection on the ability of relevant vector species to transmit any of these viruses has not been determined (Ruckert *et al*., 2017).

The findings indicate that *Ae. aegypti* is an important mosquito in the area and that the study has provided evidence of Dengue virus and Chikungunya virus prevalence in wild-caught mosquitoes at the human-livestock-wildlife interface in central Tanzania**.** A previous study in Kilosa district has reported high prevalence of DENV and CHIKV among febrile children (Chipwaza *et al*., 2014). A study conducted at human-livestock-wildlife interfaces in Kenya established presence of DENV in mosquitoes (Musa *et al*., 2020).

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. These findings provide an important step for further identification of potential areas that might influence host-vector interactions and ultimately the emergence of mosquito-borne virus epidemics.

The phylogenetic analysis and BLASTn results of the DENV-3 structural polyprotein region obtained in the present study indicated that it has 100 percent nucleotide identity to DENV 18XN10607 (Accession number MK894339.1) which is a DENV 3 isolated from China (Su *et al*., 2019) and (Accession number KX812511.1) that was isolated in 2013

from a DENV infected patient in Kenya (Konongoi *et al*., 2016). Studies conducted, reported the sequence homology among the DENV-3 strain isolated from the traveler to Tanzania (GenBank accession no. AB549332) and isolate from Tanzania was similar to those in viruses circulating regionally and the Middle East (Moi *et a*l., 2010).

Disease could have been introduced or reintroduced into the country from neighboring areas through movement of people from Kenya and China during business and tourism. Travelers increase the risk for DENV introduction from endemic to non–disease-endemic areas where competent vectors such as *Ae. aegypti* or *Ae. albopictus* mosquitoes are present.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, this study has shown that arbovirus vectors are prevalent in the interface of human-livestock-wildlife ecosystem in Kilosa, Morogoro. The species collected have been documented as potential vectors previously. CHIKV and DENV detected provide insight that pose a high risk of arbovirus transmission in these areas due to presence of these potential vectors, and their close proximity to humans. It is worth to carry out prevalence studies to determine the infection among human population. It is equally important to establish surveillance programme to monitor arboviral infection risk in the area. Immediate control measures could include public education and how to deal with key mosquito breeding sites such as use of larvicide as a vector control measure.

6.2 Recommendations

The high abundance of mosquito vectors along the interface of human-livestock-wildlife ecosystem in Kilosa calls for public education campaign on the medical and veterinary importance of these vectors as this could reduce the risk of exposure. There is the need to enhance continuous surveillance of arbovirus activity in humans, animals and vector along the interface of human-livestock-wildlife ecosystem in Kilosa to avoid maintenance of the virus in mosquitoes that may lead to future outbreaks. Integrating arboviral screening/diagnosis at point of care should be encouraged to improve diagnosis of febrile illnesses among the population in the area. There should be proper disposal of water holding containers to minimize breeding grounds for *Aedes aegypti* in particular, as well as other mosquito species. Education on environmental management and control of mosquito breeding sites should be emphasized.

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APPENDICES

Appendix 1: Mosquito survey form

Collection type	Host	Larval habitat	Water movement
Immature	Human	Pond-Lake	Stagnant
Resting mosquito from	Horse	Ground Pool	Slow
House	Cow	Swamp	Moderate
Animal Shelter	Other	Marshy Depression	Fast
Cave	**ENVIRONMENT**	Stream Margin	**TURBIDITY**
Tree hole	Rain Forest	Stream Pool	Clear
Vegetation	Evergreen Forest	Rock Pool	Coloured
Other	Cloud Forest	Seepage-Spring	Turbid
Biting/Landing mosquito from	Coniferous Forest	Ditch	Polluted
Human	Scrub/Bush	Well	**PHYICAL FACTORS**
Animal	Savanna	Artificial Container	pH
Type Net	Prairie	Hoof Print	Conductivity
Light Trap	Island	Rut	Temperature
Bait Trap	Swap	Rice field	**AQUATIC VEGETATION**
Swarming	Salt Marsh	Mangrove	Submerged
At light	Beach	Other	Floating
BG sentinel	Mangrove	**ALGAE**	Emergent
Aspirator	Orchard Plantation	filamentous	Floating and Emergent
other	Cultivated field	Green	Submerged Emergent
TERRAIN	Bamboo Grove	Brown	Submerged Emergent
Mountain	Urban	Other	All types
Hill	Village	**ALGAE DENSITY**	**QUANTITY OF AQUATIC VEG**
Valley	Other	None	None
Plateau	**ENVIRONMENTAL MODIFIERS**	Scarce	Scarce
Plain	Primary	Moderate	Moderate
DISTANCE FROM HOME meters	Secondary	Abundant	Abundant
SKY	Agriculture	**DIMENSION OF SITE**	**ARTIFICIAL CONTAINER**
Clear	Pasture	Area	Types
Partly cloudy	Grove/Plantation	Depth	Size(volume)
Overcast	Other	**WATER**	Location
Fog	**WIND**	Permanent	Use
Mist	None	Temporary	
Light Rain	Light	**SALINITY**	
Heavy Rain	Gusts	Fresh	
	Strong	Brackish	
	HEIGHT ABOVE GROUND		

Appendix 2: Extraction of viral RNA (modified qiagen protocol)

Procedure

1. Preparing the samples:
    i. Thaw the homogenised lysate/sample on ice.
    ii. Vortex the sample to mix well
2. Pipette 560 µL prepared Buffer AVL containing carrier RNA into a 1.5 ml microcentrifuge tube.
3. Add 140 µL of sample to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.
4. Incubate at room temperature (15–25°C) for 10 min (Longer incubation times have no effect on the yield or quality of the purified RNA).
5. Briefly centrifuge the tube to remove drops from the inside of the lid.
6. Add 560 µL ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
7. Carefully apply 630 µL of the solution from step 6 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.
8. Carefully open the QIAamp Mini column, and repeat step 7. Repeat this step until all of the lysate has been loaded onto the spin column.
9. Carefully open the QIAamp Mini column, and add 500 µL Buffer AW1. Close the cap, and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
10. Carefully open the QIAamp Mini column, and add 500 µL Buffer AW2. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Continue directly with step 11, or to eliminate possible Buffer AW2 carryover, perform step 10 and then continue with step 11.
11. Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µL Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min.
12. Centrifuge at 6000 x *g* (8000 rpm) for 1 min. Perform a double elution using 2 x 40 µL Buffer AVE.
13. Aliquot eluted RNA into 4 aliquots of 20 µL each while maintaining cold temperature using Ice Park.
14. Store at –80°C for future use.

Appendix 3: Ethical clearance certificate

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20th November 2019

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RE: ETHICAL CLEARANCE CERTIFICATE FOR CONDUCTING MEDICAL RESEARCH IN TANZANIA

This is to certify that the research entitled: "Mosquitoes at the interface of human-livestock-wildlife ecosystem in Kilosa, Tanzania" (Machelle IS. 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, Community Development, Gender, Elderly & Children 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, Community Development, Gender, Elderly & Children 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 as per NIMR Act No. 23 of 1979, PART III Section 10(2).
5. Sites: Mikumi National Park Ecosystem Morogoro region.

Approval is valid for one year: 20th November 2019 to 19th November 2020.

Name: Prof. Yunus Daud Mgaya

Signature
CHAIRPERSON
MEDICAL RESEARCH
COORDINATING COMMITTEE

Name: Prof. Muhammad Bakari Kambi

Signature
CHIEF MEDICAL OFFICER
MINISTRY OF HEALTH, COMMUNITY DEVELOPMENT, GENDER, ELDERLY & CHILDREN

CC: Director, Health Services-TAMISEMI, Dodoma
RMO of Morogoro region
DMO/DED of Kilosa district

Appendix 4: Pools screened for arboviruses infection in Aedes mosquitoes

Location	Pool number	DENV	CHIKV	YFV	RVFV
Mikumi	1	–	+	–	–
Mikumi	2	–	+	–	–
Mikumi	3	–	+	–	–
Mikumi	4	–	+	–	–
Mikumi	5	–	+	–	–
Mikumi	6	–	–	–	–
Mikumi	7	–	+	–	–
Ihombwe	8	–	+	–	–
Ihombwe	9	–	–	–	–
Ihombwe	10	–	+	–	–
Ihombwe	11	–	–	–	–
Lumango	12	+	+	–	–
Lumango	13	–	–	–	–
Lumango	14	–	–	–	–
Lumango	15	–	–	–	–
Lumango	16	–	–	–	–
Lumango	17	–	–	–	–
Lumango	18	–	–	–	–
Ihombwe	19	–	–	–	–
Lumango	20	–	–	–	–
Lumango	21	–	–	–	–
Lumango	22	–	–	–	–
Lumango	23	–	–	–	–
Lumango	24	–	–	–	–
Lumango	25	–	–	–	–
Lumango	26	–	–	–	–
Lumango	27	–	–	–	–
Lumango	28	–	–	–	–
Lumango	29	–	–	–	–
Lumango	30	–	–	–	–
Lumango	31	–	–	–	–
Lumango	32	–	–	–	–
Lumango	33	–	–	–	–
Lumango	34	–	–	–	–
Lumango	35	–	–	–	–
Lumango	36	–	–	–	–