Investigation of Mosquito (Diptera: Culicidae)-Borne Viruses Circulating in some selected areas of Lusaka District, Zambia.

By

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A Dissertation submitted to the University of Zambia in partial fulfillment of the requirements for the award of the degree of Master's in Tropical Infectious Diseases and Zoonosis

The University of Zambia

Lusaka

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DECLARATION

I, YUSUF ESHIMUTU ABU, declare that I have written this dissertation and that the work has not been submitted for any other degree or professional qualification to any other institution. Where assistance was sought, it has been acknowledged accordingly. It has been presented by the guidelines for the Masters of Science dissertation of the University of Zambia.

Signature …….. Date…….

CERTIFICATE OF APPROVAL

The University of Zambia approves the dissertation submitted by YUSUF ESHIMUTU ABU, as fulfilling the partial requirements for the award of the Master of Science Degree in Tropical Infectious Diseases and Zoonosis by the University of Zambia

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ABSTRACT

Mosquito-borne viruses are RNA viruses cutting across different virus families and genera of emerging and re-emerging viruses that account for millions of disease incidences annually worldwide. Since the circulation of viruses in vectors heralds an outbreak of diseases, this present cross-sectional study was undertaken from February to April 2022 to investigate the presence of mosquito-borne viruses in captured mosquitoes from six selected residential areas of Lusaka. The Mosquitoes were collected using CDC light traps both indoors and outdoors supplemented with CO_2 from yeast fermentation. The mosquitoes were morphologically identified and some were confirmed using mitochondrial cytochrome c oxidase subunit 1 DNA Barcoding. Female (2074) mosquitoes were pooled according to their genera and sampling locations. A total of 71 pools containing a maximum of 40 mosquitoes each were screened using a pan-flavivirus, pan-alphavirus, and pan-phlebovirus RT-PCR assay to detect the respective virus genera genome in total RNA extracted from the mosquito lysates. Phylogenetic analysis based on RNA-dependent RNA polymerase (RdRp) amino acid sequences was used to characterize the detected viruses. Three mosquito genera capable of transmitting arboviruses were identified in this study namely *Aedes*, *Anopheles*, and *Culex* mosquitoes. *Aedes aegypti*, *Anopheles rufipes*, and *Culex quinquefasciatus* were confirmed by DNA Barcoding. Three of the 71 pools tested positive for Phlebovirus and none of the pools was positive for either flavivirus or alphavirus. Only one positive sample was successfully sequenced and detected in a pool of *Culex quinquefasciatus* collected in Kanyama. The blast result from the NCBI website showed an 85.69% and 97.45% identity with Culex bunyavirus 2 for nucleotide (blastn) and protein (blastp) respectively. The detected virus showed the virus is closely related and clusters with Culex bunyavirus 2 and Culex bunya-like virus which are unclassified bunyaviruses forming a separate clade from other classified bunyaviruses. This study provides the first evidence of the circulation of Culex bunyavirus 2 among mosquitoes in the selected parts of Lusaka District. Further studies should be conducted to determine the potential of the detected virus in causing disease in humans or animals. In addition, similar studies should be conducted in other parts of Zambia to determine the presence of the virus.

DEDICATION

I dedicate this piece of work to my replica and young brother Damian Eshimutu Abu who always asks when I shall return home.

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ACRONYMS AND ABBREVIATIONS

-	Negative
%	Percentage
˚C	Degree Celsius
+	Positive
<	Less than
>	Great than
~	Approximately
µl	Microliter
Ae	*Aedes*
An.	*Anopheles*
bp	Base pair
CDC	Centre for disease control and prevention
CHIKV	Chikungunya virus
CO_2	Carbon-dioxide
COXI	Cytochrome c oxidase subunit 1
Cx	*Culex*
DENV	Dengue virus
DHF	Dengue hemorrhagic fever
DNA	Deoxyribonucleic Acid
DSS	Dengue severe syndrome
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic reticulum
FAO	Food Agriculture Organization

Fig.	Figure
G	Glycoprotein
GPS	Geographic Position System
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Kb	Kilobase
kDa	Kilo Dalton
L	Large segment
M	Medium segment
ml	Millilitres
Mn.	*Mansonia*
mRNA	Messenger ribonucleic Acid
MWAV	Mwinilunga alphavirus
NC	Negative control
Nm	Nanometre
NS	Non-structural
NW	New World
ONNV	O'nyong- nyong virus
ORF	Open reading frame
OW	Old World
PCR	Polymerase Chain Reaction
PRNT	Plaque Reduction Neutralization Test
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction Reverse transcription polymerase chain reaction
RdRp	RNA-dependent RNA polymerase

RNA	Ribonucleic Acid
rpm	Revolution per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction Reverse transcription polymerase chain reaction
RVFV	Rift Valley Fever Virus
S	Small segment
US EPA	United State Environmental Protection Agency
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow fever virus

CHAPTER ONE

1.0. INTRODUCTION

1.1. Background to the Study

Arboviruses are RNA viruses cutting across different virus families and genera of emerging and re-emerging viruses that account for millions of disease incidences annually worldwide. Arboviruses are transmitted to humans and animals by arthropods such as mosquitoes, ticks, sandflies, and midges threatening the health of both animals and humans (Mbanzulu et al., 2017). Viruses transmitted by mosquitoes are referred to as mosquito-borne viruses and are responsible for widespread morbidity and mortality worldwide including Sub-Saharan Africa (Mazaba-Liwewe et al., 2014). The family of viruses transmitted by mosquitoes includes the *Togaviridae* (genus: Alphavirus), *Flaviviridae* (genus: Flavivirus), and the *Bunyaviridae* (genus: Orthobunyavirus and Phlebovirus) (Braack, *et al.*, 2018). The transmission patterns and life history of these viruses are the same making their prevention and control common (Jones et al., 2020) with only a subtle difference.

The viruses circulating in mosquitoes cut across several families and could be further classified as those transmitted to other hosts and insect-specific/restricted viruses. Mosquito-borne viruses' host range includes both warm and cold-blooded animals such as fish, amphibians, reptiles, mammals, and birds causing severe diseases (Hermanns et al., 2020). There has been a worldwide discovery and detection of Insect-specific viruses (ISVs) in different mosquito species (Wastika et al., 2020). The inability of insect-specific viruses to infect vertebrate cells and the absence of reports on disease causation limits interest to study them. On the other hand, ISVs have been known to possess the potential of disrupting pathogenic arbovirus transmission and reducing vector competence. This has contributed to the understanding of biodiversity and the complexity of vector-borne viral diseases in nature. Hence, the interest in recent times to study them (Blitvich and Firth, 2015; Supriyono et al., 2020).

Clinical appearance typical of arbovirus infection comprises a vague febrile sickness that might include arthralgia, rash, and joint pain, with or without neurological or hemorrhagic disorders. The diagnosis of the disease depends on the knowledge of its transmission,

clinical manifestation, and other differentials, like blood count to give valuable insights (Eckerle et al., 2018).

Arbovirus infections go unnoticed in malaria-endemic regions such as Lusaka, as febrile illnesses are often misdiagnosed as malaria. These have the potential to lead to large outbreaks of arbovirus diseases and treatment failure (Mbanzulu et al., 2017). The existence of mosquito-borne diseases in a community is dependent on the interaction between the virus, reservoir, vector, and susceptible host in a favorable environment (Brugueras et al., 2020), hence the need for continuous surveillance of the diseases.

Lusaka is experiencing rapid urbanization with a fast-growing population of over 2 million people, this will increase the likelihood of disease spread and the far-reaching impact of zoonoses. An increase in population densities has been linked to rising in disease outbreaks due to close contact with the vector and increased vectors (mosquito) activity arising from the development of habitat and breeding sites. This is especially so for container mosquito vectors such as *Aedes* and the common household mosquito (*Culex* species) which continue to thrive in expanding urban environments (Gould and Higgs, 2009; Braack et al., 2018). In addition, international travel, and importation of goods from other countries such as tires are frequent infective sources and vector sources to other areas. Furthermore, increased ecosystem disruption and transformation have enhanced contact between infected wildlife or sylvatic vectors and humans which have also been reported to contribute to the emergence of arbovirus diseases (Braack et al., 2018).

Aedes mosquitoes and *Culex* mosquitoes are major arboviral vector species globally; and are known for Chikungunya, Zika, Dengue, and West Nile virus transmission (Jones et al., 2020). *Aedes aegypti* has a wide distribution in the tropics and subtropical region of the world and is a major vector in urban arboviruses transmission due to multiple blood-feeding habits per day unlike other mosquitoes with only a one-time feeding pattern (Girard et al., 2020).*Culex* mosquitoes are also known for the transmission of West Nile virus (WNV). The cycle of the disease is maintained between the *Culex* mosquitoes and wild birds in the course of feeding (Bellini, *et al.*, 2014).

To prevent outbreaks of arbovirus diseases, early surveillance is required in vectors before animal/human cases arise. Seroprevalence studies in Zambia have shown substantial evidence of a wide range of arboviral infections in humans (Mweene-Ndumba et al., 2015; Chisenga et al., 2020) and in non-human primates (Wastika et al., 2019). The isolation of

the West Nile virus in Zambia (Orba et al., 2018) in mosquitoes also confirms the presence of arboviruses. A novel alphavirus, tentatively named Mwinilunga alphavirus (MWAV), was also discovered in North-Western Province (Torii et al., 2018) from *Culex* mosquitoes. The presence of arboviruses in neighbouring countries like Tanzania, Angola, Democratic Republic of Congo (Bisimwa, 2016; Mbanzulu et al., 2017) makes the continuous surveillance of arboviruses necessary for early detection.

1.2. Statement of the Research Problem

Mosquito-borne viral disease outbreaks indicate insufficient mosquito-borne pathogen investigation as mosquito-borne viruses are most likely to be heralded by virus-bearing mosquitoes in the field. Field mosquitoes are colonized by viruses and spread in nature leading to an outbreak (Fang et al., 2021). Arboviruses have a wide geographical spread due to the presence of invasive mosquito vectors among vulnerable or susceptible hosts. Aside from mortality and morbidity caused by mosquito-borne viruses to humans, outbreaks have a dire socioeconomic impact as regards livestock abortions, morbidity, and death (Wright et al., 2019).

The growing population size and urbanization of Lusaka may cause changes in the equilibrium of mosquito vectors due to the activities of man. This increases the chances of outbreaks and epidemics by providing a suitable environment for mosquitoes to thrive (Mweene-Ndumba et al., 2015). There has been little attention to the distribution and abundance of mosquito species in Lusaka as only a few studies have been conducted (Masaninga et al., 2012). This may ultimately compromise observing the presence of new species or non-recorded species in the nation which may transmit diseases (Adugna et al., 2021).

The neglect to examine the incidence of arboviruses in their possible vectors may lead to large epidemics as the diseases are mostly misdiagnosed as malaria and typhoid as a result of generic clinical manifestations that can delay final diagnosis in resource-poor countries including Zambia (Eckerle et al., 2018).

1.3. Significance of the study

The world is suffering from a succession of arboviral disease outbreaks, hence the need to better comprehend the current dissemination and potential future spread of their vectors for effective monitoring and control programs. Thus, this study will provide knowledge on mosquito-borne viruses circulating in some selected parts of Lusaka district.

Detecting viruses in circulation among potential vectors will provide knowledge of arbovirus disease status in some selected parts of Lusaka District which could be useful for modelling transmission dynamics and help in the mapping of disease distribution.

This will enhance preparedness to develop appropriate and sustainable control and preventive measures to reduce outbreaks as the detection of arboviruses in mosquitoes will serve as an early warning for appropriate action.

1.4. Study Objectives

1.4.1 General Objectives

To detect medically important mosquito-borne viruses circulating in mosquitoes in selected parts of Lusaka District.

1.4.2. Specific Objectives

The specific objectives are

i. To identify various mosquito species collected from selected parts of Lusaka district.
ii. To detect Mosquito-borne viruses from the collected mosquitoes.
iii. To phylogenetically characterize medically important arboviruses detected in collected mosquitoes.

1.5. Research questions

What are the species of mosquitoes circulating in some selected parts of Lusaka District of Zambia?

What are the possible viruses transmitted by the mosquito species circulating in selected parts of Lusaka district of Zambia?

What are the strains of arboviruses circulating in selected parts of Lusaka district of Zambia transmitted by mosquitoes?

CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Mosquitoes (Diptera: Culicidae)

Mosquitoes are common insects distributed worldwide in tropical regions with hot and humid climates. Mosquitoes belong to the order Diptera, suborder Nematocera, Family Culicidae and sub-families Anophelinae, Culicinae, and Toxorhynchitinae, with 34 genera and over 3100 species which *Anopheles, Aedes and Culex* mosquitoes are mostly involved in the spread of human disease (Tennyson and Jayakumar, 2020).

Care must be taken when identifying mosquitoes as they can be mistaken for other similar insects. Mosquitoes are easily recognized due to their distinct morphology (Figure 2.1) which comprises being slender, and long-legged, and the presence of scales and long proboscis that separate them from other insects. An adult mosquito has all of the following three distinct features:

i. a pair of wings;
ii. scales are found on the veins of the wings and a fringe of scales on the hind edge (magnification will be required to see these veins and the scales)
iii. a long proboscis (mouth part) extending from the head and many times longer than the head itself (WA Department of Health, 2022).

The immature stages are also distinguishable from other aquatic insects by the absence of legs, and among the subfamily due to their respiratory apparatus. Anophelinae possesses posterior anal papillae and a pair of respiratory openings while the subfamily Culicinae has an elongated siphon near the end of the abdomen. However, they share similar characteristics such as head-bearing mouth brushes and antennae, and a bulging thorax that is broader than the head and abdomen. Mosquitoes are usually, and most reliably, identified as mature (fourth-instar) larvae and adults (Bradford, 2005). Owing to the similarity among males of various generic-level taxa, females are needed to distinguish many species. A great effort is required to distinguish closely related species into separate monophyletic lines due to their obvious similarity (Bradford, 2005; Harbach, 2007).

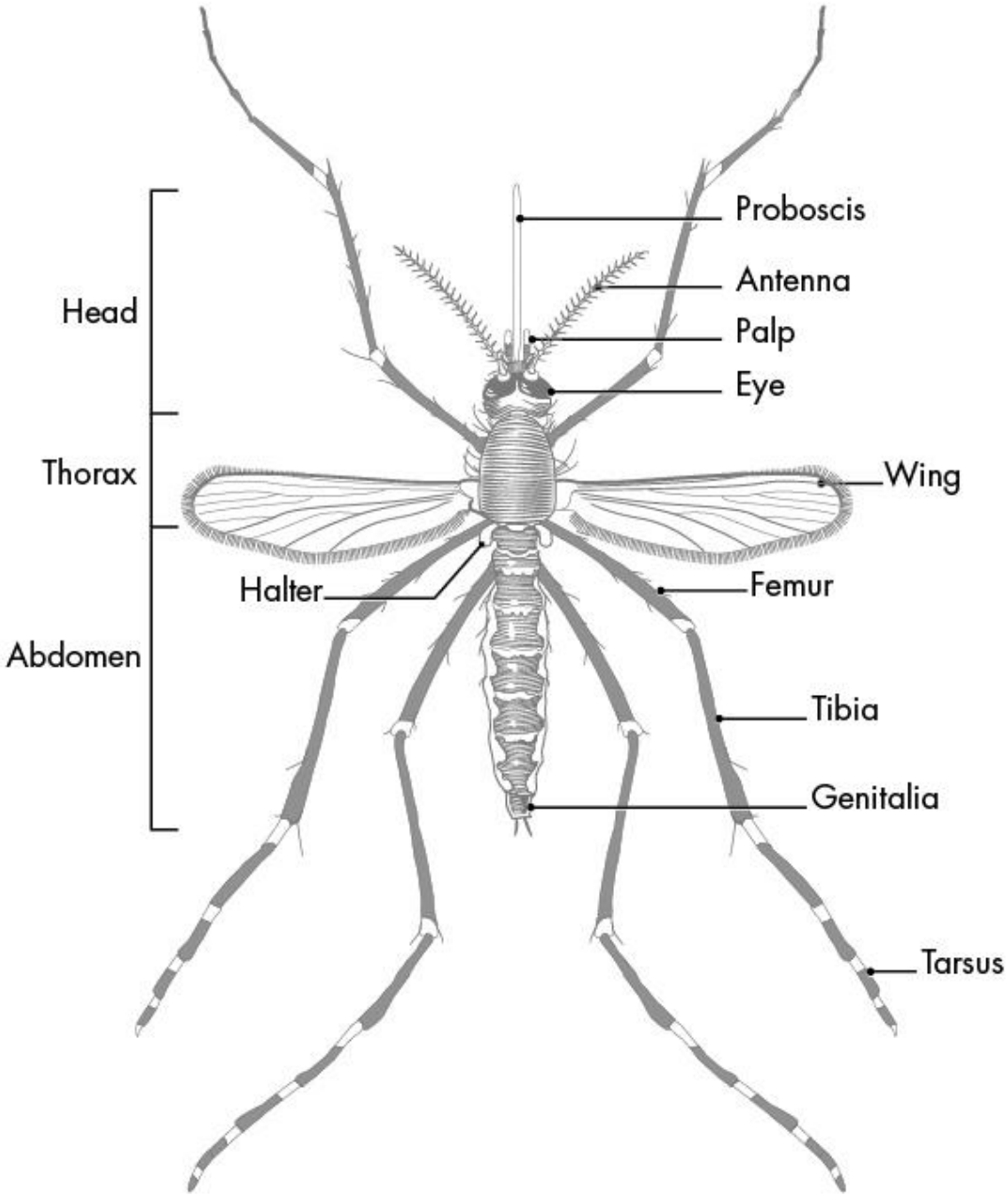

Figure 2.1: Dorsal view of adult female *Aedes aegypti* mosquito (Source: CDC 2020).

Mosquitoes exhibit a holometabolous life cycle that takes place in both terrestrial and aquatic environments (Figure 2.2). The juvenile stages of mosquitoes inhabit a range of aquatic environments. Eggs are normally laid by adult mosquitoes on or near natural and artificial water-carrying sources such as fruit shells and husks, plastics bottles, rock holes, groundwater, crab holes, leaf axils, bamboo internodes, fallen leaves and spathes, flower bracts, crab holes, snail shell, bromeliads and aroids, and pitcher plants. The eggs under suitable conditions hatch to larvae also known as "wriggler" and metamorphose to pupae also known as "tumblers" subsequently and remain in the aquatic environment until

emergence as an adult in the terrestrial habitat (Bradford, 2005; Harbach, 2007). The duration to hatch depends on the type of mosquitoes, nutrients available, and water temperature. The larva is an active stage of the mosquito lifecycle, characterized by feeding and developing into the dormant stage "Pupa" which remains in water without feeding until it emerges as an adult. The period of emergence to adulthood ranges from two to three days in the aquatic environment and completes their development outside the aquatic environment (Bradford, 2005; Harbach, 2007; US EPA, 2021).

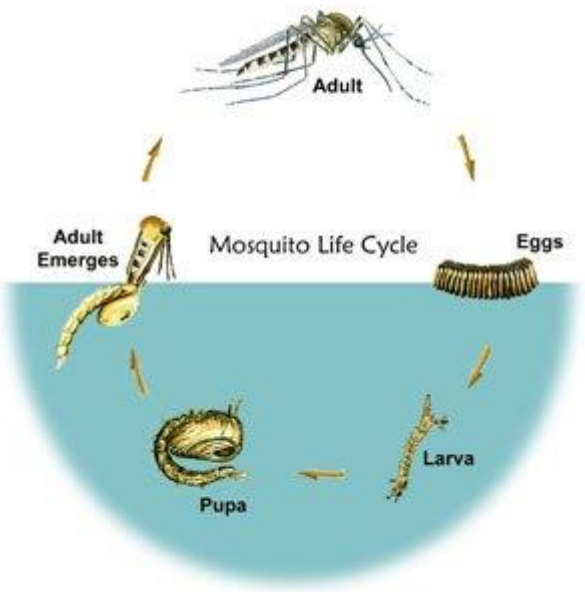

Figure 2.2:Life Cycle of Mosquito (Source: US EPA, 2021)

Mosquitoes' activities are at their peak mostly when there is high humidity and moderately cool air. Mosquitoes feed mainly on fluids from plants, including fruit juice, exudates, nectar, and honeydew. In addition, females require blood meals from animals for egg development, though some species do not require a blood meal. Most species occupy spaces within a few meters to the ground and others are found in the forest canopy, especially in the wild. The source of the blood meal is usually warm-blooded vertebrates. In addition, some species feed on insects, including lepidopterous larvae, nymphal cicadas, mantids, and other ectotherm snakes, turtles, frogs, and toads. Some species fly and feed mostly during the night (nocturnal) and twilight (crepuscular). While others fly and feed mostly during the daylight hours (diurnal) (Harbach, 2007).

Some factors contribute to the wide distribution and population of mosquitoes, these include rainfall, temperature, type of mosquitoes, and economic activities like the trading of goods (OECD, 2018). Other environmental factors relating to the landscape are

imperative in determining mosquito abundance and composition such as canopy cover, land cover/vegetation, elevation, soil properties, and hydrology (Johnson et al., 2020).

2.1.1 Mosquito Identification

Vector identification is paramount in the area of medical entomology for both clinical and research purposes. Over the years, different approaches to mosquito identification have emanated other than traditional morphological identification, this includes the use of proteomics tools, isozyme analysis, and PCR, and nucleic acids sequencing (Jourdain et al., 2018). The taxonomy of mosquitoes is of great significance, as the incrimination of vectors to transmit a pathogen depends on the proper identification of the vector (Batovska et al., 2016; Gunathilaka, 2017). Morphological identification remains the standard method for both routine surveillance and research purposes. This is an inexpensive method, easy to apply, and requires little technical equipment in the field. The morphological identification method is prone to some limitations such as being time-consuming, requiring skilled workers, and sometimes key features used for identification are missing from the mosquitoes due to handling, preservation, and storage. Hence, other methods are becoming relevant (Jourdain et al., 2018).

2.1.2 Distribution of Mosquitoes in Zambia

Masaninga *et al*., (2016) reported the occurrence of *Aedes aegypti* in peri-urban areas and *Aedes africanus* in forested areas of the North-western Province in Zambia. This mosquito genus has the potential to carry viruses of medical importance. Another study conducted from May to July 2003 in two peri-urban locations of Chazanga and Kalikiliki in Lusaka district showed a high prevalence of Culicine mosquitoes (Chanda et al., 2012). Another study conducted in Lusaka in 2009, reported high larvae densities of *Culex quinquefasciatus* mosquito in sewerage maturation ponds, and a large number of adult *Cx. quinquefasciatus* indoors and up to 2,000 *Cx. quinquefasciatus* in an out-door open structure (Masaninga, *et al*., 2012). The presence and abundance of these WNV-competent vectors are a cause for concern in Lusaka. Several species of *Culex* mosquitoes have been identified in Zambia, these include *Culex (Culex) quinquefasciatus Say, Culex (Culex) univittatus* Theobald, *Culex (Culex) antennatus*(Becker), *Culex (Culex) poicilipes* (Theobald), *Culex (Oculeomyia) bitaenorhynchus* Edwards, *Culex (Culiciomyia) nebulosus* Theobald, *Culex (Lutzia) tigripes* DeGrandpré & DeCharmoy reported from Macha (Kent, 2006).

2.1.3. Mosquitoes capable of diseases transmission

Mosquitoes have been referred to as public health enemies due to their ability to transmit and spread diseases such as filariasis, dengue, haemorrhagic fever, encephalitis, yellow fever, chikungunya, and malaria. Furthermore, the discomfort they cause to human through bites and noise lead humans to sleeplessness and irritation (Adugna, *et al.*, 2021). The mosquito genera incriminated for disease transmission include *Aedes*, *Anopheles* and *Culex* mosquito

2.1.3.1. *Aedes* Mosquitoes

Aedes aegypti and *Aedes albopictus* are widely known container mosquitoes. They are efficient daytime biters; hence frustrate control methods such as bed nets that prevent evening/night-time biting mosquitoes from contact with humans. Since preventing bites during the day is tricky compared to the night they tend to effectively transmit diseases to humans (ECDC, 2017)

Aedes aegypti is an anthropophilic mosquito native to Africa and the main vector for dengue, Zika, yellow fever, and chikungunya viruses worldwide. The mosquito species is divided into two subspecies: *Ae. aegypti aegypti and Ae. aegypti formosus* found worldwide in subtropical and tropical regions and sub-Saharan Africa respectively (Dickson *et al.*, 2014;Mcgregor and Connelly, 2020).

Though morphologically *Ae. aegypti* males and females are similar, generally, males are smaller and possess plumose antennae while females' palps are shorter compared to males (OECD, 2018). *Aedes aegypti* was first incriminated as a vector for arbovirus in 1900 in Cuba and its ability to transmit DENVs by Walter Reed, Carlos Finlay and James Carroll in 1901. In addition, Thomas Bancroft in 1906 linked the incidence of DENVs transmission to the daytime biting habits of *Aedes aegypti*. The concept of vector control was borne from understanding the role of mosquitoes in the spread of human pathogens, hence, the control of pathogen transmission through the control of vectors (Souza-Neto et al., 2019)

Ae. aegypti have been associated with recent outbreaks of mosquito-borne viruses in the United States of America, comprising dengue outbreaks in 2010 and 2013, a chikungunya outbreak in 2014, and the 2016 outbreak of Zika virus (McGregor and Connelly, 2021). The *Ae. aegypti* possess features that allow it to be an efficient arbovirus vector as they are particularly anthropophilic and live inside or around human dwellings and prefer to bite humans. The capability of *Ae. aegypti* to transmit pathogens has been demonstrated by its

multiple host-seeking and feeding within a single gonotrophic cycle. Hence, enhancing the acquisition and spread of pathogens (Scott et al., 1993) and the preference for blood meals over sugar meals as a source of energy (McGregor and Connelly, 2021). Another unique feature of *Aedes aegypti* is its ability to successfully infiltrate and establish in new areas and the ability to lay eggs in many containers per gonotrophic cycle due to the skipping oviposition habit enables them to be widespread (McGregor and Connelly, 2021).

Aedes (Stegomyia) *albopictus* (Skuse), is native to Southeast Asia, islands of the Western Pacific, and the Indian Ocean. Over the years, it has spread to other regions including America, Africa, the mid-east, and Europe, and further extended during the early 20th century eastwards across Pacific islands. This has led to the concern that the mosquitoes have the potential of causing serious outbreaks of arbovirus diseases (Gratz, 2004).

Laboratory studies have confirmed many arboviruses transmission by *Ae. albopictus* to laboratory animals and birds, and have also been isolated from wild-caught mosquitoes of this species accounting for at least 22 arboviruses, especially in the Americas. Hence *Ae. albopictus* is a competent vector and continues to spread, replacing *Ae. Aegypti* populations in some areas with anthropophilic tendencies (Gratz, 2004).

2.1.3.2 *Culex* Mosquitoes

Culex mosquitoes are nicknamed the southern house mosquitoes and they are the most abundant house mosquitoes that develop in polluted and standing waters in cities and towns, they are also found in street gutters, polluted tanks, water bottles, tin cans, polluted ponds, ornamental ponds, ditches, creeks, barrels, and marshes in subtropical and tropical countries (Tennyson and Jayakumar, 2020). *Culex pipiens pipiens, Culex pipiens quinquefasciatus, and Culex tarsalis* are among the *Culex* spp. considered to be the most significant and capable vectors of West Nile virus (WNV) (Diaz-Badillo et al., 2011; Tennyson and Jayakumar, 2020).

Morphologically, *Culex* mosquitoes have shorter palpii than the proboscis. The lateral and dorsal sides of the thorax and the abdominal segments are marked with scale patterns that are unique and helpful in the identification of *Culex* mosquitoes. Ornamentation is very rare. The scutellum in the thorax is trilobed. Pale and dark bands are prominent in the abdominal segments. The resting position of the adult mosquito is parallel to the surface (Tennyson and Jayakumar, 2020).

Species of the *Cx. pipiens* complex particularly *Culex quinquefasciatus* has established itself due to swift unplanned urbanization creating a suitable environment for its development in the tropical and subtropical world enhancing their proliferation. The establishment of *Culex quinquefasciatus* in or near a human settlement or activity increases the chance of depending on humans or mammals and avian hosts for a blood meal (Nchoutpouen et al., 2019). The larvae are normally found in polluted man-made or artificial containers of water including wet pit latrines, open ponds, septic tanks, ditches, and drains containing human or animal sewage (Masaniga, 2012; Nchoutpouen *et al*., 2019).

Culex pipiens is a pest in urban environments of Europe which has led to an organized effort to control this complex species since the early 20th century. The species exhibit high ecological plasticity, which depicts their feeding behaviour and vectorial capabilities. The females of *Cx. pipiens* have been implicated for Usutu Viruses and West Nile virus transmission in Europe, the diverse host preference enhances the amplification cycle of the pathogen, especially for West Nile virus (WNV) in birds, filarial worms (canine dirofilariasis), plasmodia that cause avian malaria and also the sporadic spill-over of several other arboviruses to human and other mammalian hosts (ECDC, 2020).

Soh and Aik, (2021) demonstrated how variation in weather can be used to envisage the abundance of *Culex* mosquitoes and the potential risk of *Culex*-borne flavivirus spread in urban regions in Singapore. They reported an increase in *Culex* larval activity with higher temperatures in non-residences and a decline in activity with increased rainfall in residences. Therefore, they suggested that priority be given to *Culex* mosquito control when temperatures are rising, and drier weather is on the way to reduce the risk of *Culex*-borne flavivirus transmission.

2.1.3.3. *Anopheles* Mosquitoes

Anophelines are mosquitoes known for transmission of arboviruses, protozoan and Dirofilaria nematodes of veterinary and medical importance. *Anopheles is* the only mosquito genus known to spread the *Plasmodium* parasite that causes malaria in humans (Gunathilaka, 2017). However, not all of the *Anopheles* species are capable of malaria transmission due to their numbers and infrequent contact with humans (WHO, 2007). *Anopheles* genus is frequently reported as ‘complex’ a cluster of species that are nearly identical morphologically but then considered distinct. In other cases, they are reported as “group” species that are nearly the same as an adult but many can be separated at their

immature stages or using molecular methods (Coetzee, 2020) Such groups include the renowned *Anopheles gambiae* complex (*An. gambiae, Anopheles coluzzii, Anopheles arabiensis, Anopheles fontenillei, Anopheles bwambae, Anopheles melas, Anopheles merus, Anopheles quadriannulatus, Anopheles amharicus*); *Anopheles coustani/crypticus/namibiensis* in southern Africa; *Anopheles marshallii* complex (*An. marshallii, Anopheles letabensis, Anopheles hughi, Anopheles eleskosiensis*) and its allies *Anopheles hargreavesi, Anopheles gibbinsi* and *Anopheles mousinhoi*; *Anopheles squamosus/cydippis*, the former of which is known to consist of at least five chromosomal forms; *Anopheles funestus* group (*Anopheles funestus, An. funestus-like, Anopheles leesoni, Anopheles rivulorum, An. rivulorum-like, Anopheles brucei, Anopheles parensis, Anopheles vaneedeni, Anopheles aruni, Anopheles confusus, Anopheles fuscivenosus*);*Anopheles nili* complex (*An. nili, Anopheles somalicus*) (Coetzee, 2020)

Studies have confirmed that *Anopheles* mosquitoes spread and maintain the O'nyong-nyong virus one of the alphaviruses in the family *Togaviridae* transmitted to humans. The anthropophilic nature of *Anopheles* suggests constant exposure to arboviruses in human blood meals, especially in endemic regions. This may increase the chance of *Anopheles* to transmitting other arboviruses in endemic zones. The perception that anophelines are intrinsically less capable of arbovirus transmission than culicines requires more studies to establish the biological basis for this phenomenon (Minkeu and Vernick, 2018).

Several arboviruses and insect-specific viruses have been detected or isolated from Anophelines aside from O'nyongNyong Virus (ONNV) having the capacity to cause diseases cutting across Alphaviruses like Sindbis Virus (SINV), Semliki Forest Virus (SFV), Eilat Virus (EILV), Venezuelan Equine Encephalitis Virus (VEEV), and Western Equine Encephalitis Virus (WEEV); Flavivirus such as Japanese Encephalitis Virus (JEV), Wesselsbron Virus (WSLV), Anopheles Flavivirus (AnFV), West Nile Virus (WNV), Haslams Creek Virus (HaCV), Dairy Swamp Virus (DSwV), Mac Peak Virus (McPV), Long Pine Key virus (LPKV), Kampung Karu Virus (KPKV), Anopheles gambiae Flavivirus (AngFV), Anopheles squamosus Flavivirus (AnsFV), Karumba Virus (KRBV), Stratford Virus (STRV); and Phlebovirus like Rift Valley Fever Virus (RVFV) (Minkeu and Vernick, 2018).

2.2. Mosquito-borne viruses (Arboviruses)

Arbovirus is the term referred to as arthropod-borne viruses. The viruses are spread among different viral families (Figure 2.3). These viruses rely on the vertebrate host and arthropods like mosquitoes, ticks, sand flies, bugs, and midges for their survival and replication (Kolawole et al., 2018). Mosquito-borne viruses remain common in tropical zones like Lusaka and spread within and to non-endemic areas as a result of rapid urbanization, extensive deforestation, and change in land use. In addition, the risk of transmission of arboviruses and mosquitoes may be exacerbated in Lusaka as a result of increased international travel and trade (Ferede et al., 2018).

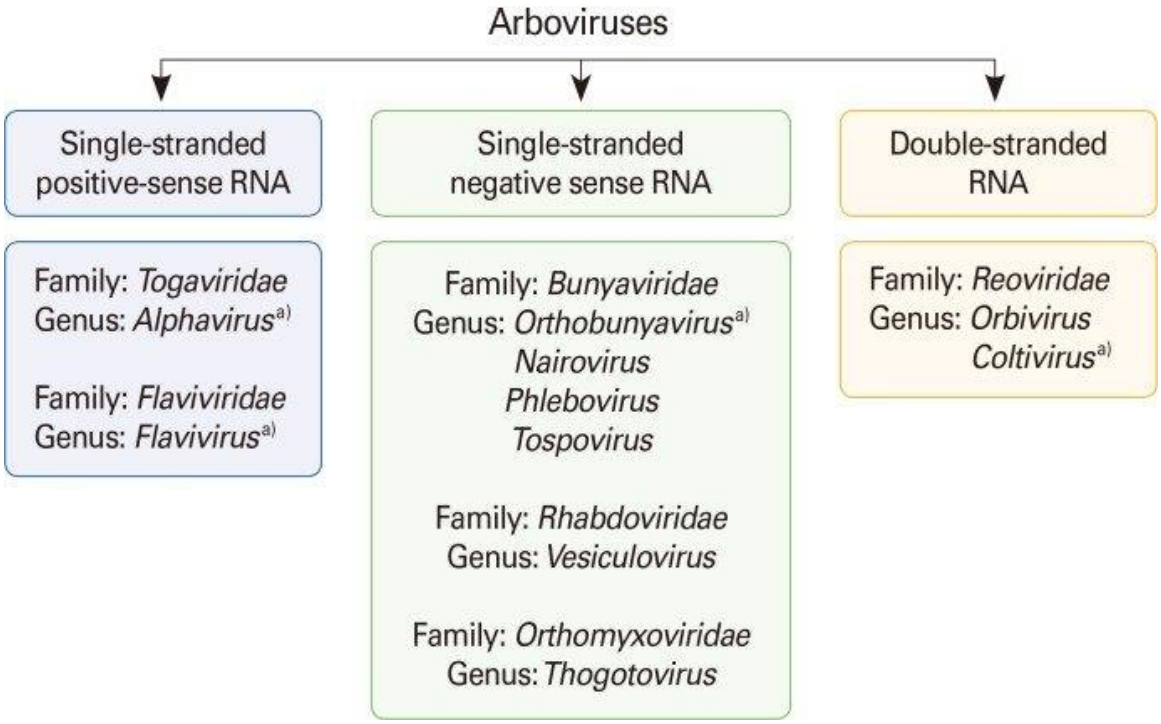

Figure 2.3:Classification of Arboviruses (Source: Go et al., 2014)**.**Arboviruses are included in six different taxonomic virus families. Arboviruses that cause human encephalitis belong to four genera in four virus families indicated with an 'a)' superscript

Numerous factors have been identified to contribute to the spread of arboviruses in tropical countries. Among these factors, the temperature has been shown to contribute to vector competence, and the ability of vectors (mosquitoes) to carry and spread diseases. An increase in temperature speed up the reproductive capacity of mosquitoes leading to a high mosquito population, in turn increasing the likelihood of disease transmission. The mosquito population, the types of the species, and the nature of the arbovirus strain contribute to the mosquito's ability to transmit viruses (Ciota and Keyel, 2019). Other factors that facilitate mosquito-borne virus outbreaks aside from temperature include

rainfall and humidity as outbreaks have occurred frequently during the rainy season (Ferede et al., 2018).

2.2.1 Alphaviruses

Alphaviruses are among the mosquito-borne viruses known to cause infection in humans and animals belonging to the virus family *Togaviridae*. These viruses are known to have a very wide geographic distribution except in Antarctica and many islands. Each species of the virus have a limited distribution (Strauss and Strauss, 1994) and have been reported to cause disease in large numbers of people.

Alphaviruses are divided into two sub-groups centred on the clinical manifestation and geographic distribution of these viruses. The New World (NW) or encephalitic alphaviruses are responsible for neurological effects and the Old World (OW) or arthritogenic alphaviruses are responsible for the rheumatic disease (Rao and Taylor, 2021).

Alphaviruses are small, enveloped viruses, ∼70 nm in diameter, having a single-stranded, positive-sense, RNA genome approximately 12 Kb in length (Leung et al., 2011).. Alphaviruses are structurally similar and share a common lifecycle. The virion comprises a nucleocapsid core enclosed by a host-derived lipid bilayer enclosed with transmembrane glycoproteins, E1 and E2, which are arranged in trimeric spikes of heterodimers on the surface of the virion (Figure 4.4). The non-structural proteins (nsPs) are encoded at the 5' end of the genome, while structural proteins are encoded at the 3' end. The structural proteins are translated from a subgenomic RNA, while the nsPs are translated from genomic RNA (Galán-Huerta et al., 2015). Mosquito-borne alphaviruses responsible for the human rheumatic disease are widely distributed and include Ross River virus, chikungunya virus, Barmah Forest virus, O'nyong-nyong virus, Sindbis virus, and Mayaro virus (Rangel and Stapleford, 2021)

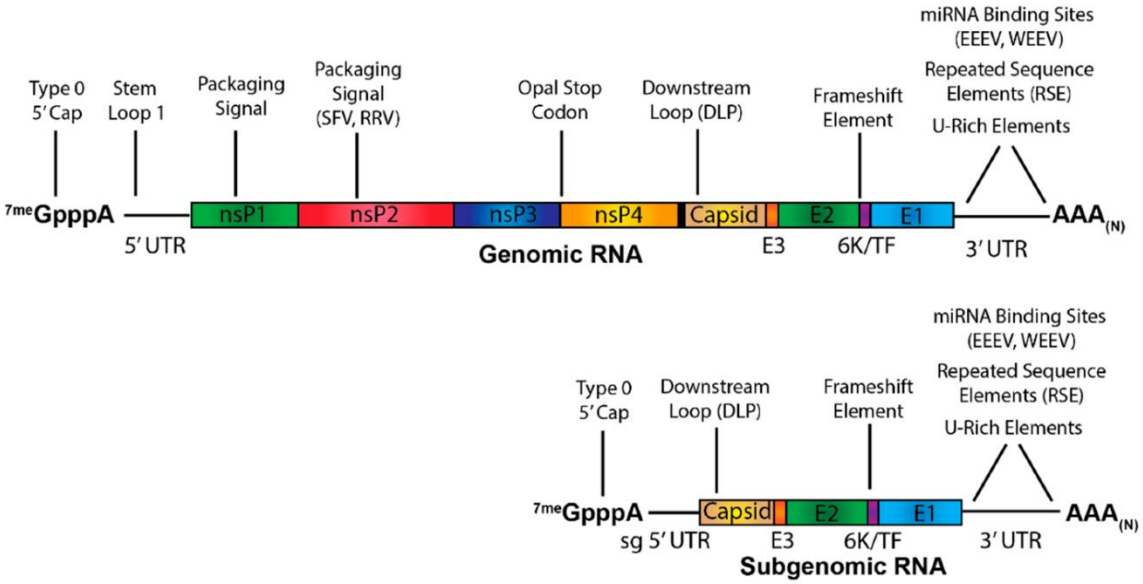

Figure 2.4:Alphavirus Genome Organization (Source: Lapointe and Sokoloski, 2021). Alphaviral Genetic Organization and RNA Virulence Traits. Sequences and secondary RNA structures in the genomic and subgenomic RNA known to impact virulence are indicated. Elements that have only been shown to be present in a subset of alphaviruses have the specific viruses listed in parentheses. sg 5′UTR = subgenomic 5′UTR.

Alphaviral diseases are associated with nonspecific symptoms which generally run from weeks to months such as fever, myalgia, rash, polyarthritis, polyarthralgia, and sometimes lead to debilitating conditions like encephalitis and myocarditis leading to death (Rangel and Stapleford, 2021; Suhrbier et al., 2012). Failure of the immune response to clear infection and persistence of the virus in the tissues leads to chronic alphaviral rheumatic diseases (Suhrbier *et al.*, 2012).

The diagnosis of Alphaviruses may involve recovering the viral particle during acute infection usually 2–4 days during symptom manifestation from patient blood and pharyngeal swabs in case of Venezuelan equine encephalitis virus(VEEV). CHIKV and VEEV RNA have also been detected directly from serum and both serum and pharyngeal swabs respectively (Weaver and Smith, 2011). However, serodiagnosis remains the standard diagnostic method (Suhrbier et al., 2012), and its availability is often restricted only to some regions and may not be found in areas where they are needed. Several serologic tests are available such as immunofluorescence, ELISA, neutralization, and heamagglutination inhibition test (Weaver and Smith, 2011).

Simple analgesics and Non-steroidal anti-inflammatory drugs (NSAIDs) are the available treatment options, which provide relief to the pain associated with the disease. There is a need to develop drugs that will cure diseases.

2.2.1.1 Chikungunya Virus

Chikungunya is one of the mosquito-borne viral diseases caused Chikungunya virus (CHIKV) that is responsible for numerous epidemics globally and has rapidly spread into new geographical areas over the last two decades (Mascarenhas et al., 2018). Chikungunya virus caused 1.4–6.5 million cases during an epidemic between 2004–2011 and led to imported cases reported in nearly 40 countries (Suhrbier et al., 2012). CHIKV is an alphavirus in the family *Togaviridae*. The name of the virus emanated from the Makonde people of Tanzania in their native language "chikungunya" meaning "bent over in pain" describing the bent posture of infected individuals due to excruciating joint pains and arthralgia that forces the person to bend (Cunha et al., 2020; Mascarenhas et al., 2018)

Chikungunya virus is an icosahedral enveloped virus with a genome of around 12 kb, positive sense single-stranded RNA, and a diameter of 70 nm having similar genome organization as other alphaviruses (Figure 2.4). Phylogenetic analysis of 2005 CHIKV outbreak isolates in Reunion Island revealed that the isolates evolved into the East/Central/South African (ECSA) lineage, with more recent Indian Ocean strains creating an Indian Ocean sub-lineage within ECSA (Mascarenhas et al., 2018). The ECSA has recently spread in Brazil causing abnormal outcome of the disease involving high cases and severe neuropathies (Cunha et al., 2020) The West African lineage of CHIKV originated in Africa and then travelled to Asia, where it evolved into a unique Asian lineage (Mascarenhas et al., 2018).

The first CHIKV epidemic and isolation was reported from 1952 to 1953 from a patient in Tanganyika Province of Tanzania during an epidemic of a dengue-like disease that had been circulating in that zone(Cunha et al., 2020). There have been recurrent reports of this in earlier years in other parts of Africa, including Mozambique (Mascarenhas et al., 2018) and Zambia, CHIKV was first described in 1961 in Luanshya, Copperbelt Province (Rodger, 1961). Ever since the first case, no report of the disease until a 36.9% seroprevalence from Lukanga Swamp residents in Central Province of Zambia (Chisenga et al., 2020). Human outbreaks of chikungunya have also been reported in Zimbabwe and South Africa. The first case was reported in the eastern part of the Northern Province in 1956, 1975/769, and 1977 and Southern Zimbabwe in 1961/62 and 1971. Furthermore, in 2001 one case of CHIKV was identified in an individual who had lived in the eastern region of the Northern Province of Zimbabwe. An epizootic in monkeys with no human cases has also been reported in 1964 in northern KwaZulu Natal, South Africa (Jupp, 2005).

The spread of CHIKV occurs from a bite of infected female mosquitoes (*Aedes aegypti* or *Aedes albopictus*), and recently, maternal–foetal transmission was documented (Schwartz and Albert, 2010). The transmission of the virus involving mosquitoes, and its circulation has been associated with a sylvatic cycle where enzootic transmissions between non-human primates and *Aedes* spp. mosquitoes, such as *Ae*. (Stegomyia) *africanus*, *Ae*. (Diceromyia) *taylori, Ae.* (Diceromyia) *furcifer*, *Ae.* (Stegomyia) *luteocephalus,* and *Ae.* (Stegomyia) *neoafricanus,* sporadically spilled over to humans and in the urban cycle where humans and *Ae. Albopictus* and *Ae*. a*egypti* are involved (Cunha et al., 2020).

CHIKV disease is non-fatal and self-limiting, it usually clears within 5–7 days. However, for reasons that are still unknown but are being investigated, the recent outbreaks of the diseases are associated with more severe symptoms and increased death cases in young children, elderly and immunocompromised individuals. CHIKV infection has a negative impact on the socioeconomic productivity and quality of life of infected individuals as a result of developing debilitating conditions such as tenosynovitis, polyarthralgia, and severe tendons and joint pain that could last for several months or years in over 30% of infected individuals (Cunha et al., 2020). Severe haemorrhage and shock are seldom observed but localized pain in joints and tendons is commonly observed(Galán-Huerta et al., 2015).

Several approaches have been employed in the detection of chikungunya virus disease. Serologically, antibodies are detected using enzyme-linked immunosorbent assays (ELISA) to ascertain the presence of IgG and IgM anti-chikungunya antibodies in serum. IgM antibody can be detected for about 8 weeks and its levels are at a peak between 3 and 5 weeks post-infection (WHO, 2020). The CHIKV can also be detected directly from blood using reverse transcriptase–polymerase chain reaction (RT–PCR) during the first few days of infection. Genotyping of the virus and characterization of the virus are further carried out from the product of RT–PCR from clinical samples for comparative study among patients and geographical locations (WHO, 2020).

Despite the debilitating effect of the disease, there is no specific and effective antiviral therapy available. Vaccines are still undergoing trials. Protection against mosquito bites and vector control remains the only effective preventive measures. Clinicians need to differentiate chikungunya fever from dengue fever and other diseases to give effective treatment and prevent the disease from spreading (Galán-Huerta et al., 2015). This absence of specific medications or licensed vaccines to treat or prevent CHIKV disease, in addition

to the presence of mosquito vectors, contributes to the worldwide presence of the disease which makes it a public health concern (Cunha et al., 2020).

2.2.1.2 Mayaro Virus

Mayaro virus (MAYV) is an arbovirus that is endemic in tropical forests in South and Central America, particularly in the Amazon basin. It was first isolated from forest workers in Mayaro County in Trinidad and Tobago in 1954 (Anderson et al.,1957). There has been concern regarding the invasion capacity of MAYV into urban areas to cause epidemics throughout the region. Sporadic outbreaks have been reported since the initial isolation of the virus in 1954 from the region causing febrile illness (Caicedo et al., 2021). Few cases have been reported in other regions as a result of increased transnational travel and tourism from people in Europe and North America to the endemic region (Dieme et al., 2020; Caicedo et al., 2021).

Mayaro virus (MAYV) is responsible for Mayaro fever, it is an enveloped RNA virus containing a single-stranded genome of around 11.5 kb sequences. The virus possesses a complex life cycle involving *Hemagogus* mosquitoes and animals including birds, rodents, reptiles, horses and other non-human primates. MAYV is an alphavirus in the family *Togaviridae* (Caicedo et al., 2021). Mayaro virus (MAYV) shares commonality with Chikungunya virus and is classified in the antigenic complex of Semliki Forest viruses with other alphaviruses (Bebaru, O'nyong-nyong, Ross River, Semliki Forest,Getahand and Una) of medical and veterinary importance (Ganjian and Riviere-Cinnamond, 2020; Izurieta et al., 2018). Alongside the Una virus (UNAV), MAYV is considered as a new world member of the Semliki forest antigenic complex. Evolutionary analysis has described D (widely dispersed), L (limited), and N (new), with limited geographic distribution based on vector habitat and host range (Caicedo et al., 2021).

MAYV is sustained in an enzootic cycle through constant evolutions and transmission between daytime mosquitoes from the forest canopy to birds, nonhuman primates, rodents, and marsupials with human infection occurring due to spill over from the enzootic cycle (Ganjian and Riviere-Cinnamond, 2020). The mosquitoes implicated in the transmission of MAYV are *Haemagogus janthinomys* and studies showed competence also in *Aedes aegypti, Aedes scapularis, Culex* sp., *Psorophora* sp, *Sabethes sp., Anopheles quadrimaculatus* and *Coquillettidia* sp (Izurieta et al., 2018; Pereira et al., 2021) making the spread of the virus to urban areas increasing. Some of these mosquitoes have also been

detected in Zambia and other neighbouring countries (Masaninga et al., 2014; Bisimwa et al., 2016; Orba et al., 2018).

So far, only minute studies have been reported in Africa for the Mayaro virus (Velu *et al.*, 2021). In Zambia, the first and only study was in Central Province reported the seroprevalence of 19.6% of Mayaro virus among Lukanga Swamp Residence (Chisenga et al., 2020). This indicates the presence of the Mayaro virus circulating in Zambia.

Though no studies have shown infected individuals to be asymptomatic, the clinical manifestation of Mayaro fever tends to resemble Chikungunya virus infection like other alphaviruses which are generic and include retro-orbital pain, vomiting, myalgia, high-grade fever, incapacitating arthralgia, headache, maculopapular rash, diarrhoea, and prolonged development of arthralgia (Ganjian and Riviere-Cinnamond, 2020).

Diagnosis of MAYV is mainly based on symptoms and lack of specific tests and cross-reactivity with other arbovirus makes diagnosis difficult which leads to underreporting of cases. Methods such as neutralization and reverse-transcription polymerase chain reaction (RT-qPCR) tests have been employed. Molecular detection of MAYV in plasma and urine samples is becoming promising in the diagnosis of MAYV (Pereira et al., 2021).

To date, there is no approved treatment or vaccine due to low research funding, and inconsistent or lack of surveillance has contributed to the upsurge of MAYV circulation. However, a minimum of three vaccine candidates have been established. There is a need for urgent development of therapeutics and increased surveillance to halt, prevent, and predict future epidemics to ensure control measures are in place (De O'Mota et al., 2019; Caicedo *et al.*, 2021). For early detection and control, preventive measures including mosquito surveillance are cardinal (Dieme et al., 2020).

2.2.1.3 O'nyong-nyong Virus

O'nyong'nyong virus (ONNV) is known to be transmitted by *Anopheles gambiae* and *Anopheles funestus* through their bite. The virus belongs to the Alphavirus genus in the family *Togaviridae*. The virus was first isolated in Uganda in 1959 and was named by the Acholi people of North-western Uganda based on the symptom of the disease as 'o'nyong-nyong,' describing the 'very painful weakening of joints,' (Williamset al., 1965). The virus is restricted to the African continent and is responsible for many outbreaks especially in Eastern and Western regions of Africa. Some of the sub-Saharan Africa countries have experienced outbreaks like Nigeria, Democratic Republic of Congo, Central African

Republic, Cameroon, Senegal, Kenya, Uganda, Tanzania, Malawi, and Mozambique (Bessaud et al., 2006; Pezzi et al., 2019; Rezza et al., 2017).

ONNV is closely linked to members of the Semliki Forest antigenic complex that cause arthritis and belongs to the genus Alphavirus in the family *Togaviridae* (Rezza et al., 2017). O'nyong'nyong virus is positive sense, single strand RNA virus. It is enveloped and spherical about 60 nm in diameter, and the nucleocapsid is about 40 nm in diameter and holds 240 copies of the capsid protein. The genome size is approximately 11.8 kb with a 5' cap and a 3' poly-A tail. The two-third portion of the 5' genome encodes 4 non-structural protein genes and the other third of the genome encodes 3 structural protein genes, which are translated from subgenomic mRNA (Figure 5) (Marceau and Moore, 2016; Saxton-Shaw et al., 2013). O'nyong-nyong is a genetically unique virus, it is closely related to CHIKV as compared to other arthritogenic alphaviruses. Like its close relative CHIKV, o'nyong-nyong virus has 45–68% attack rate (Suhrbier et al., 2012).

Evolutionary studies showed ONNV and CHIKV belong to the same monophyletic group within the Semliki Forest complex, where ONNV isolates form a separate clade away from all CHIKV strains, suggesting that the divergence occur at least thousands of years ago. Phylogenetic studies suggest that the same virus is circulating in Africa, this was clearly described in a study that showed ONNV strains isolated from Gulu, Uganda, in 1959 and Senegal in 1963, being closely related and the formation of separate clades among strains from Nigeria (1966), Uganda (1996), and Chad (2004) from the analyses of partial genome sequences available from the Genbank confirmed the two major clade (Bessaud et al., 2006).

The cases of ONNV have been underreported and/or misdiagnosed probably because of other pathogens circulating in Africa causing comparable illnesses such as chikungunya virus (CHIKV), dengue virus (DENV), *Plasmodium falciparum*, and other arboviruses. The problem is compounded as a result of cross-reactivity between antibodies against CHIKV and those against ONNV. ONNV fever is associated with severe arthralgia similar to chikungunya fever except for cervical lymphadenitis. Other manifestations of ONNV infection are fever, joint pains (without effusions), headache, posterior cervical lymphadenopathy, mainly in the large joints, conjunctivitis (red eyes), and often itchy maculopapular skin rash. Furthermore, bleeding gums or nosebleeds rarely occur (Pezzi et al., 2019; Rezza et al., 2017).

The symptoms are self-limiting and there is no treatment or effective vaccine against the disease. However, animal models suggest that the CHIKV candidate vaccine may confer protection against ONNV. The Preventive measures include sleeping under an insecticide treatment net to prevent the bite from anopheles mosquitoes similar to those adopted against malaria parasite transmission (Rezza et al., 2017).

2.2.2 Flaviviruses

Flaviviruses are spherical enveloped viruses. They are single-stranded positive (+) sense RNA genome of around 9.7–12 kb and approximately 50 nm in diameter. The genome encodes three structural (C, prM, and E) and seven non-structural (NS) proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Figure 2.5). The surface proteins of the viral capsid are arranged in an icosahedral-like symmetry (Agboli et al., 2021). They possess a monopartite and linear genome. Despite this similarity in structures, flaviviruses have fundamental differences in their transmissibility and host range (Blitvich and Firth, 2015). These viruses can be divided into four main categories: (i) mosquito-borne flaviviruses, (ii) insect-specific flaviviruses (ISFs), (iii) tick-borne flaviviruses, and (iv) no known vector (NKV) flaviviruses (Daidoji et al., 2021). Most recognized flaviviruses are known as dual-host viruses as they are transmitted horizontally between blood-sucking arthropods and vertebrate hosts (Blitvich and Firth, 2015). Flaviviruses are major human pathogens that use diseases in a high number of humans annually posing a public health concern in many countries. They include dengue, tick-borne encephalitis, Japanese encephalitis, yellow fever, West Nile, and Zika viruses responsible for a wide range of human diseases including fever, hemorrhagic fever, encephalitis and microcephaly (Barrows et al., 2018). In contrast, insect-specific flaviviruses (ISFs) are known to infect only arthropods and no such virus has been isolated from vertebrates. These ISFs have a unique feature as they are known to affect the multiplication of pathogenic flaviviruses when they co-infect mosquito cells. Hence, block the pathogenic virus transmission to humans. Therefore, it is important to pay attention to both ISFs and human-pathogenic flaviviruses, even though ISFs appear not to have a direct harmful effect on human wellbeing (Daidoji et al., 2021),

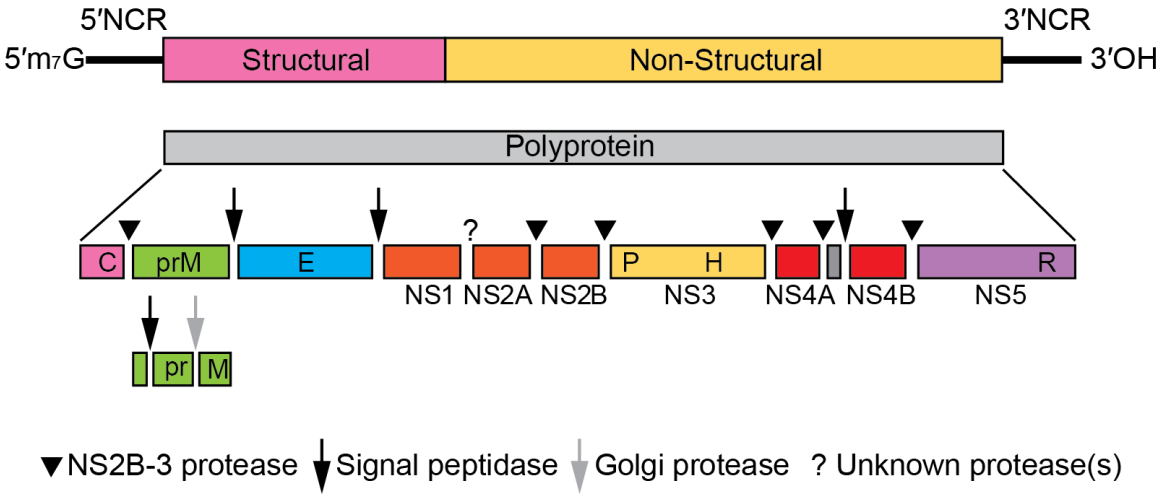

Figure 2.5: Flavivirus Genome Organization (Source: ICTV, 2022). Flavivirus genome organization (not to scale) and polyprotein processing. The virion RNA is about 11 kb. At the top is the viral genome with the structural and nonstructural protein coding regions and the 5′- and 3′-NCRs. Boxes below the genome indicate viral proteins generated by the proteolytic processing cascade. P, H, and R symbols indicate the localization of the NS3 protease, the NS3 RNA helicase, and the NS5 RdRp domains, respectively.

Most of these flaviviruses are transmitted by mosquitos, and their spread is exacerbated by increased temperature, human population growth, and movement, and the spread of vectors (Barrows et al., 2018). The viruses are mostly transmitted to their hosts by blood-seeking mosquitoes or ticks. Nonetheless, sexual and transplacental routes have also been documented (Araujo et al., 2020).

Most flaviviruses cause a variety of febrile syndromes subject to the viral strain and the status of the affected individual. Human flavivirus infections can be asymptomatic or present nonspecific symptoms such as body aches, fever, headache, and joint pain(Araujo et al., 2020). Some of these infections may also lead to vomiting and diarrhoea in the first few days usually 2-15 days after infection, (Musso and Desprès, 2020). Some of the flaviviruses are also classified as zoonotic viruses (Araujo et al., 2020).

On one hand, a number of flaviviruses are neurotropic such as West Nile (WNV), Usutu virus (USUV), Zika virus (ZIKV), Japanese encephalitis virus (JeV), Ilheus Virus (ILHV), and Tick-borne encephalitis virus (TBeV) can spread to the spinal cord and brain to cause severe neurological syndromes such as, meningitis, acute flaccid paralysis and encephalitis. On the other hand, flaviviruses such as DeNV YFV, and ZIKV cause visceral disease resulting in haemorrhagic syndromes, liver failure and vascular compromise. ZIKV has been described to be transmitted sexually resulting in placental insufficiency, congenital

malformations, and microcephaly, which may result to severe complications leading to foetal death (Pierson and Diamond, 2020).

The well-established and routinely used method for detecting the flaviviruses is the RT-PCR method by designing primers that detect conserved regions in the NS5 region of the flavivirus genome among many diverse species of the viruses (Daidoji et al., 2021). This nucleic acid detection using real-time RT-qPCR or conventional RT-PCR to detect flavivirus-associated human illnesses has proved reliable (Hoyos-López et al., 2016). Serological assays are also used in clinical settings and are commercially available. Some of the available serological techniques include complement fixation and hemagglutination inhibition assay which has replaced the former commercial immunofluorescence assays, enzyme-linked immunosorbent assay and the immunochromatographic lateral flow strip tests (Musso and Desprès, 2020).

2.2.2.1 West Nile Virus

The West Nile virus is among the earliest detected arboviruses known to be responsible for disease in man. The virus was first isolated in 1937 from the blood of a female having fever in the West Nile province of Uganda (Monath, 1989).Taxonomically West Nile virus (WNV) belongs to the Japanese encephalitis virus (JEV) serocomplex in the flavivirus genus. It maintenance and amplification is dependent on the presence of passeriform birds and *Culex* mosquitoes in nature. *Culex* mosquitoes are the sole vectors of WNV. Nonetheless, *Aedes* spp and *Ochlerotatus* spp could also serve as vector to a lesser extent, while a number of bird species act as amplifying host with outbreaks caused due to spill over or tangential transmission to humans and equids that serve as dead-end host (Weaver and Reisen, 2010).

West Nile Virus particles have a single-stranded positive-sense RNA genome. The genome comprises of a single open reading frame ofapproximately11 kb and lack polyadenylation tail at the 3' end (Colpitts et al., 2012). The viral capsid is composed of a single protein (capsid protein C) of icosahedral typed covered by a smooth an enveloped made of the envelope E and the membrane M. The WNV genome encodes for both structural proteins and non-structural proteins genes totalling into 10 genes within the 5′ portion of the ORF (open reading frame) contains C, prM and E and within the 3′ portion contains NS1, NS2A, NS2B,NS3, NS4A, NS4B and NS5 respectively (Figure 2.5)(Bahuon et al., 2015).

West Nile virus (WNV) is a mosquito-borne neurotropic Flavivirus endemic in Africa, Europe, the Middle East and Western Asia. Mosquitoes acquire the virus during blood from infected birds, the reservoir hosts of the virus. People are then infected when they are bitten by these virus-infected mosquitoes leading to disease in humans with symptoms ranging from mild fever to severe meningoencephalitis (Bahuon et al., 2015).Most infected people are asymptomatic, some manifest symptoms including headache, joint pains, body aches, diarrhoea, vomiting, and rash, as well as severe impairment to the central nervous system in some infected individuals, resulting to meningitis, encephalitis, and occasionally death (US EPA., 2016).

There is no recorded outbreak of West Nile virus in Zambia. However, there has been serological confirmation of WNV in Zambia. The first and only study of WNV in humans in the country was reported in 2015, with a prevalence in human of 10.3% (Mweene-Ndumba et al., 2015). Other reports of WNV in Zambia include detection of WNV in *Culex* mosquitoes (Orba et al., 2018) and in farmed crocodiles at a prevalence of 6.7% (Simulundu *et al.*, 2020). The detection of WNV in mosquitoes from Western Province of Zambia confirmed the circulation within the communities and the detection of the virus in crocodiles in Southern Province suggest that WNV could be a neglected emerging pathogen and could be contributing to febrile illnesses in animals and humans in Zambia. Hence, the need for proper understanding of the epidemiology of viral infections and constant surveillance in Zambia (Velu et al., 2021).

Genetic characterization studies have revealed that WNV is distributed globally, with two main genetic lineages: Lineage 1 is highly invasive and widely distributed and Lineage 2 appears to be restricted to Africa (Weaver and Reisen, 2010), and both lineages have been reported in Zambia. The presence of lineages 1a was reported in farmed crocodile (Simulundu et al., 2020) and lineage 2 from *Culex quinquefasciatus* mosquitoes collected in the Western Province of Zambia and was closely related genetically to WNV lineage 2 South African strains that was previously described to be extremely neuroinvasive (Orba et al., 2018). Other Lineages of West Nile have been reported in Central Europe comprising of lineage 3 and 4, nevertheless their taxonomic position and clinical significance are unclear. In addition, lineage 5 seems to be restricted to India. Lineage 1 strains has been responsible for most severe outbreaks to date. Three subclades have been identified in lineage 1 and include, 1a that is widespread in Africa and the Mediterranean, 1b (Kunjin

virus) that is confined to Australia, and 1c that is found in Central Asia through the central highlands of India (Bahuon et al., 2015; Weaver and Reisen, 2010).

The diagnosis of WNV infection relies on clinical manifestation and immune-responses from infection. The widely used diagnostic method is IgM-capture enzyme-linked immunosorbent assay (ELISA) (Kemmerly, 2003). However, care must be exercised in diagnosis as recent immunization with yellow fever or Japanese encephalitis vaccines may result in false positive results when testing for IgM antibody against WNV. In addition, cross-reactivity from St. Louis encephalitis, dengue, and other arboviruses has also been documented, though a four-fold change in neutralizing antibody titre is required to provide a precise diagnosis of WNV infection (Kemmerly, 2003). There are no treatments or vaccines available that are approved by the Food and Drug administration (FDA)(CDC, 2021). The available therapeutic options against WNV are generally supportive (Colpitts et al., 2012). Thus far, there is no WNV-specific treatment to reduce the injury or oedema caused, although ribavirin in high doses and interferon-α 2b proved to be efficient against the WNV infection (Kemmerly, 2003).

2.2.2.2 Dengue Virus

Over the last few decades the world has been experiencing an increase in the incidence of dengue fever (Simo et al., 2019), making the dengue virus an important infectious agent with over a 30-fold increase in world-wide incidence despite the under-reporting of cases and misclassification (Otu et al., 2019). It has been reported in tropical and subtropical counties with 3.9 billion people susceptible to infection in 128 countries with close to 400 million cases reported. About 25% of the cases account for 96 million people who showed clinical signs and 22,000 of the infected people die annually globally(Roy and Bhattacharjee, 2021).

The cases of DENV has been increasing in Africa since 1980 due to lack of appropriate surveillance with reported activities mainly in East Africa and a major epidemics reported for the first time in Somalia (1982, 1993, dengue type 2), Kenya (1982, dengue type 2), Mozambique (1985, dengue type 3) and Djibouti (1991–92, Dengue type 2) (Fagbami and Onoja, 2018). Report from a systematic review and meta-analysis in Africa revealed the pooled prevalence of DENV in apparently healthy populations was 15.6%, and 3.5%for immunoglobulins (Ig) G, IgM, and for ribonucleic acid (RNA) respectively. The pooled prevalence in populations with fever was 24.8%, 10.8%, and 8.4% (3.7–14.4) for IgG, IgM,

and for RNA respectively. Therefore, urgent attention is required for Dengue fever from researchers, healthcare workers and health policy makers (Roy and Bhattacharjee, 2021).

The first evidence of dengue fever in Zambia was from a study conducted in western and North-western Province that reported 4.1% prevalence for Dengue IgG and a risk associated to travelling to Angola among the participants (Mazaba-Liwewe et al., 2014). In a separate study conducted among Lukanga swamp resident in Central Province of Zambia reported 16.8% seroprevalence of Dengue (Chisenga et al., 2020).This clearly showed the virus is circulating in Zambia.

Dengue fever is referred to as 'break bone fever' caused by dengue virus which is transmitted by mosquitoes, an enveloped virus belonging to Flavivirus genus in the *Flaviviridae* family. It is a single stranded positive (+) sense RNA virus primarily spread by *Aedes* mosquitoes. Four antigenically distinct dengue virus serotypes have been reported with distinct genotypes (DENV-1, DENV-2, DENV-3 and DENV-4). The genome of the virus contains structural and non-structural proteins like all flaviviruses. The structural proteins include the capsid (C), membrane (M) and envelope (E) proteins, while the non-structural proteins consist of the NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The untranslated region (UTR) of DENV genome is flanked at 5′ and at the 3′ by 94 nucleotides (nts) and 388–462 nts respectively (Figure 2.3)(Otu et al., 2019; Roy and Bhattacharjee, 2021). The four DENV serotypes are classified into different genotypes as a result of the high mutation rate despite sharing 65-70% sequence similarity (Azhar et al., 2015).

The wide spread of dengue virus infection is due to mosquito vector propagation and spread, as result of globalization. On the other hand, early slave trade from Africa has been implicated to enhance the spread to other continent (Fauci et al., 2019). Other factors that exacerbated the spread include: the poor housing, overcrowding, and improper waste, water and sewer management systems that characterized unplanned towns and the non-existence of active mosquito control in dengue endemic areas (Halstead, 2017).

There are three clinical manifestations of the DENV disease raging from febrile illnesses or asymptomatic infection, mild illness to severe illness corresponding to classical dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) respectively (Otu et al., 2019). Dengue fever, is the commonest dengue infection characterized by a benign illness with symptoms like anorexia, malaise, fever, lymphadenopathy and maculopapular rash (Fagbami and Onoja, 2018).

Over the years some techniques have been employed in diagnosis of dengue virus. These includes the detection of antibodies against DENV, the detection of DENV antigens, isolation of the virus and detection of genomic sequence using nucleic acid amplification (Otu et al., 2019).

Treatment for the disease is basically supportive as there is no licenced treatment or drug. The administration of fluid and electrolyte to cushion the effect of capillary leakage syndrome due to DHF and DSS and blood in the case of haemorrhage (Fauci et al., 2019). The implementation of such therapeutics is also difficult in remote or resource-limited areas and also the use convalescent plasma or monoclonal antibodies prove to be abortive as the virus is clear from the blood before the onset of shock by the immune system. This approach may result to antibody-mediated immunopathogenesis (Fauci et al., 2019).

2.2.2.3. Zika Virus

Zika virus (ZIKV) is a mosquito-borne virus that is primarily transmitted by *Aedes* mosquitos and belongs to the flavivirus genus within the *Flaviviridae* family. In 1947, the virus was isolated from the Zika forest in Uganda. Since it was discovered, major and minor outbreaks have been documented from various parts of the world. In recent years, the virus has emerged and expanded its geographic range to various countries in Africa, Asia, Oceania, and North and South America. (Koppolu and Raju, 2018; Sharma *et al.*, 2020).

The Zika virus is an enveloped, icosahedral virus of the Spondweni clade composed of a positive sense, single-stranded RNA with a genomic size of about 11 kb and a virion diameter of about 40–60 nm. The RNA genome's single open reading frame sequence encodes a polyprotein that forms the capsid (C), membrane (M), and pre membrane portion (P) of the virus's structural architecture. There is also an envelope protein (E) and seven non-structural components (NS). These proteins are known as NS1, NS2a, NS2b, NS3, NS4a, NS4b, and NS5. The NS5 is the most highly conserved region of the flaviviruses. The NS proteins act as an RNA-dependent RNA polymerase (Figure 2.5) (Chang et al., 2016). The ZIKV is divided into two genetic lineages: African and Asian. These lineages are historically linked to the region for which they are named (Haddow et al., 2012).

ZIKV molecular and serological evidence has been reported from 26 African countries. Angola, Benin, Burkina Faso, Cabo Verde Islands, Cameroon, Central African Republic, Mali, Morocco, Mozambique, Nigeria, Côte d'Ivoire, Democratic Republic of the Congo, Ethiopia, Egypt, Gabon, Guinea-Bissau, Kenya, Niger, Senegal, Somalia, Sudan, Tanzania,

Togo, and Uganda are among the countries involved. (Adam and Jassoy , 2021). There was no information on Zika virus infection in Zambia until the study conducted in Western and Northern Province that reported the prevalence of Zika infection as 6.1% (Babaniyi et al., 2015). Furthermore, another study that used a plaque reduction neutralization test (PRNT) to quantify neutralizing antibodies found that sera from NHPs (African green monkeys and baboons) exhibited neutralizing activity against ZIKV (34.4 percent ; 33/96), despite the fact that ZIKV genomic RNA was not detected in NHP splenic tissues, implying that the presence of anti-ZIKV neutralizing antibodies represented resolved infections and that ZIKV is maintained in NHP reservoirs in Zambia (Wastika et al., 2019). In a separate study, 10.8% sero-prevalence was recorded in a Lukanga a swamp community in Central Province of Zambia for Zika in humans (Chisenga et al., 2020).

ZIKV transmission has been documented in both vector and non-vector. Several species of *Aedes* mosquitos have been implicated in vector-borne transmission, including *Aedes africanus, Aedes furcifer, Aedes taylori,* and *Aedes luteocephalus,* which act as enzootic vectors, maintaining the sylvatic cycle between mosquitos and non-human primates such as apes and monkeys (Sharma et al., 2020). The sylvatic cycle is thought to be responsible for the survival of the ZIKV lineage in Africa, but it has not been proven in Asia. Humans become unintentional hosts when bitten by forest mosquitos, and then transmit the virus to the epidemic or urban cycle in which human–mosquito–human transmission of ZIKV is observed (Agumadu and Ramphul, 2018). Transplacental transmission, blood transfusion, and sexual transmission have all been reported in non-vector human transmission. ZIKV human infection was first identified in a 10-year-old female in Nigeria (Chang *et al*., 2016), and it has since been isolated from 17 *Aedes* mosquito species as well as *Anopheles gambiae, Anopheles coustani, Culex perfuscus,* and *Mansonia uniformis* mosquitos (Koppolu and Raju, 2018).

The clinical presentation of the disease is characterized by symptoms such as joint pain, fever, headache, red eyes, and a maculopapular rash, which makes diagnosis difficult (Agumadu and Ramphul, 2018). Many infected people will be asymptomatic, and clinical illness is usually mild, lasting several days to a week, with some developing severe disease that necessitates hospitalization, which is uncommon, and deaths (Vest, 2016). There have been reports of more severe ZIKV diseases with neurological problems such as brain damage and microcephaly in foetuses born to infected mothers, as well as virus-associated Guillain-Barre syndrome in adults (Chang et al., 2016).

The detection of Zika virus infection is primarily accomplished through the detection of viral nucleic acid via RT-PCR and the detection of IgM antibodies via the IgM-capture enzyme-linked immuno-sorbent assay (MAC-ELISA) (Petersen et al., 2016). Prenatal ultrasound has been used to detect congenital Zika virus infection. However, Zika virus testing is not recommended as a preconception screening or in asymptomatic non-pregnant individuals (Agumadu and Ramphul, 2018).

Because there are no specific vaccines or antivirals available to treat ZIKV infections, prevention is the most effective way to manage and control the virus by preventing or avoiding mosquito bite. However, due to the severity of the infections, WHO designated the development of safe and effective vaccinations and novel antiviral medications as a top priority for global health, resulting in the identification of multiple vaccine and antiviral drug candidates (Koppolu and Raju, 2018; Sharma et al., 2020).

2.2.3. Phleboviruses

Phleboviruses are spread by phlebotomine sand flies and are members of the recently identified *Phenuiviridae* family in the Bunyavirales order (Sun et al., 2022). Bunyaviruses genomes are divided into three segments: L, M, and S, which encode for RNA dependent polymerase (RdRp), Gn and Gc glycoproteins (envelop proteins), and Nucleocapsid (N). They are enclosed single-stranded RNA viruses that can be negative or ambisense)(Hobson-Peters et al., 2016; Spiegel et al., 2016). Phleboviruses differ from other bunyaviruses in that they have an ambisense S segment that encodes the nucleoprotein in the negative sense and the non-structural proteins NSs and NSm in the positive sense via an overlapping open reading frame or by encoding a protein via an open reading frame (ORF). (Hobson-Peters *et al*., 2016; Elliott and Brennan, 2014). Nonetheless, both proteins are translated from subgenomic mRNAs that are transcribed from the antigenomic or genomic RNA (Elliott and Brennan, 2014)

Phleboviruses are taxonomically separated into dipteran- and tick-borne viruses transmitted mainly by eponymous Phlebotomus sandflies except RVFV associated *Aedes* and *Culex* mosquitoes and ticks respectively (Wuerth and Weber, 2016). Mosquitoes and ticks have been linked to the spread of some of the phleboviruses that pose a serious public health risk, such as Rift Valley fever virus (RVFV) and Uukuniemi virus (UUKV) (Hobson-Peters *et al*., 2016). Phleboviruses are among the new viruses that have emerged in a variety of locations around the world.

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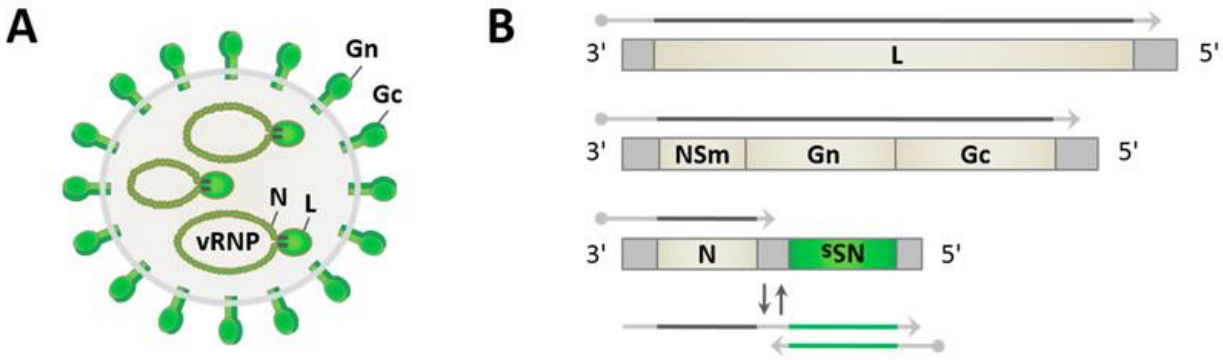

Figure 2.6: Prototypic phlebovirus virion and genome organization. (Source: Wuerth and Weber, 2016) (A) Virus particles contain the pseudo circularized tripartite single-stranded RNA genome, packaged into virus-sense RNPs (vRNPs)by nucleocapsid protein N and associated with the viral RNA-dependent RNA polymerase (RdRp) L, within a lipid envelope covered by heterodimers of glycoproteins Gn and Gc; and (B) the three viral genome segments large (L), medium (M) (both being purely negative-sense), and small (S) (ambisense)code for the structural proteins L, the Gn and Gc, and N, respectively. Viral mRNAs contain a 51-cap(dot) and short heterogeneous host-derived sequences. mRNAs transcribed from genomic RNAs are shown as grey arrows. The nonstructural protein NSs mRNA (green arrow) is synthesized from antigenomic RNA (two-coloured arrow). Dipteran-borne phleboviruses also encode a nonstructural protein on the M segment (NSm).

As a result of the viruses' tripartite genomes, the genome organization promotes genetic shift due to possible reassortment in the case of co-infection of two viruses in the same host, resulting in the development of a unique chimeric virus with a different virulence capacity (Alkan *et al*., 2013).

Several phleboviruses have been found in vectors such as sand flies, ticks, and mosquitoes. However, detection in human samples is difficult due to the overall genetic complexity and diversity of clinically important strains, as well as their predominantly nondescript clinical manifestations and a lack of awareness among some clinicians and scientists (Lambert and Hughes, 2021).The advancement in viral detection and sequencing technologies has contributed greatly in the detection and classification of phleboviruses and other viruses (Hobson-Peters et al., 2016). The phlebovirus genus comprises of 11 specific species (Lambert and Hughes, 2021) and 33 tentative named species accounting for about 70 viruses and further classified into the sand fly fever virus group and the Uukuniemi-like virus group (Elliott and Brennan, 2014)

2.2.3.1. Rift Valley Fever Virus

The Rift Valley fever virus, a phlebovirus carried by endemic African mosquitoes, is the known pathogenic mosquito-borne phlebovirus. Rift Valley fever virus causes a fatal disease in ruminants and can also cause a potentially fatal disease in humans. RVF is a World Health Organization priority infection, and it is known to be spread by over 53 mosquito species from eight genera, with *Aedimorphus* and *Neomelaniconion* mosquitos being the most common vectors (Chambaro et al., 2022). As a result of sheep deaths and miscarriages, the virus was discovered in Kenya in 1931, and it has since spread to other African countries, including the Arabian Peninsula (Chambaro et al., 2022; Stoek et al., 2022).

The Rift Valley fever virus causes nonspecific symptoms similar to those seen in most self-limiting febrile illnesses. RVFV is also one of the most dangerous human phleboviruses, causing hepatitis, haemorrhagic fever, retinal vasculitis, and encephalitis in a small percentage of cases. The Rift Valley fever virus, on the other hand, has been linked to a high rate of abortion and mortality in livestock, as well as the development of febrile illnesses in farmers who raise these animals (Lambert and Hughes, 2021).

RVF diagnosis is challenging because of its non-specific clinical symptoms, especially at the beginning of the disease. The virus can be detected in blood and other body fluids using molecular techniques such as reverse transcriptase-polymerase chain reaction (RT-PCR) and enzyme-linked immunoassay (ELISA) for antibodies. People do not get vaccines to prevent diseases because no available vaccines for humans. To avoid coming into contact with diseased animals' blood, body fluids, or tissues, people must wear protective clothes (CDC, 2020) in order to prevent infection.

2.3. Insect-Specific Mosquito-borne Viruses

Insect-Specific Viruses (ISVs) were discovered in the supernatants of *Aedes aegypti* cell lines and are known as cell-fusing agent viruses (CFAV) of the *Flavivirus* genus in the *Flaviviridae* family (Wastika et al., 2020). The viruses have since been identified in several viral taxa, including *Flaviviridae*, *Phenuiviridae*, *Mesoniviridae*, *negeviruses*, *Reoviridae*, *Nodaviridae*, *Rhabdoviridae*, and *Togaviridae* (Supriyono et al., 2020; Wastika et al., 2020).

These viruses are known as insect-specific viruses (ISVs), and they can only infect and replicate in invertebrates. They are most likely maintained by vertical transmission from

infected female mosquitoes, as several ISVs have been found in mosquito immature stages, according to some reports (Supriyono et al., 2020). Despite the fact that they have only been isolated from mosquitoes, the discovery of ISF-like sequences in other dipterans such as sand flies and chironomids suggests they may also infect other insects (Blitvich and Firth, 2015).

Because insect-specific alphaviruses are uncommon in mosquitoes and have very low prevalence rates in mosquito populations, there have been few studies on them. Only a few insect-specific alphaviruses, such as Eilat virus (EILV), Ta Forest alphavirus (TALV), Mwinilunga alphavirus (MWAV), and Agua Salud alphavirus (ASALV), have been discovered in mosquitoes from the Old World (Hermanns et al., 2020). Eilat virus, a mosquito-borne, host-restricted alphavirus, has been shown to be unable to infect vertebrates in part due to restricted RNA replication (Nasar et al., 2012). In Zambia, researchers discovered Mwinilunga alphavirus, which is related to but distinct from Eilat virus and does not replicate in mammalian cells (Torii et al., 2018).

In contrast, insect-specific flaviviruses (ISFs) have been extensively studied and classified into two evolutionary groups. Classic insect-specific flaviviruses (cISFs), which include cell fusing agent virus, Kamiti River virus and Culex flavivirus. They are phylogenetically distinct from all other known flaviviruses and number around 12 viruses at the moment. Despite their apparent insect-specific behaviour, the second group of flaviviruses is phylogenetically related to mosquito/vertebrate flaviviruses. They are not monophyletic, and the group now contains nine viruses, including Chaoyang virus, Nounané virus, and Lammi virus (Blitvich and Firth, 2015)

Culex flavivirus (CxFV), an insect-specific flavivirus with a global distribution, was discovered in *Culex pipiens* Linnaeus mosquitos in Japan. Other *Culex* species and subspecies have been discovered with additional mutations in Mexico, Uganda, Trinidad, and the United States (Newman et al., 2011).

2.4. Knowledge Gaps

In recent decades, the occurrence and impact of arboviral epidemics and the wide geographic spread of the diseases have become a serious public health concern (Girard et al., 2020). Mosquito-borne viruses in particular are known to spread between several host and mosquitoes (Hermanns et al., 2020).

Zambia being a landlocked country surrounded by other countries that have experienced outbreaks of mosquito-borne viruses predisposes the people to the possible spread of the diseases. Mosquito vectors are responsible for arbovirus transmission and are known to be invasive. Human activities such as travelling and trade have been associated with the spread of these vectors(OECD, 2018). Only a few studies have attempted to identify the various mosquito vectors in Zambia and focuses on *Anopheles* mosquitoes in region with high malaria transmission. So far, only a few studies have attempted to determine the distribution of mosquito vectors capable of transmitting arboviruses in Zambia (Kent, 2006; Masaninga et al., 2014).

Little or no attention has been given to mosquito species capable of transmitting arboviruses in Lusaka as most studies focuses on the spread of malaria (Clennon et al., 2010; Masaninga et al., 2012). Despite a report of unexpected increase in mosquito bites observed in Lusaka and complaints about mosquitoes occurring in winter and daytime biting indicating the high burden of *Culex* mosquitoes (Masaninga et al., 2012)

Several studies indicated the presence of mosquito-borne viruses in Zambia as way back as 1961 with the discovery of Chikungunya virus (Rodger, 1961) and other seroprevalence studies in both animals and humans (Babaniyi et al., 2015; Chisenga et al., 2020; Simulundu et al., 2020; Mazaba-Liwewe et al., 2014; Morita, 1988; Mweene-Ndumba et al., 2015; Wastika et al., 2019) suggesting a high burden of MAYV, YFV, ZIKV, DENV, and WNV. These studies provide the need to investigate for mosquito-borne viruses circulating in mosquitoes in Zambia as outbreaks are preceded by virus in circulation in Mosquitoes. Furthermore, the limitations from serological such as cross-reactivity owing to antibodies in the context of global co-circulation of antigenically related (re-)emerging viruses. Hence, a careful implementation and evaluation of serological testing is required to ensure a reliable diagnostics (Fischer et al., 2021).

In line with the above limitations other studies have been conducted in Zambia to detect mosquito-borne viruses circulating mosquitoes in the wild employing the Reverse transcriptase-Polymerase chain reaction to detect viruses with the potential to cause disease in humans and animals (Chambaro et al., 2022; Orba et al., 2018; Torii et al., 2018; Wastika et al., 2020). These studies detected both pathogenic and non-pathogenic viruses circulating in Zambia. However, these studies focus on other provinces of Zambia and not Lusaka.

Therefore, this study aimed to identify mosquito vectors capable of transmitting arboviruses in some selected parts of Lusaka as the rapid globalization and increase in population density of Lusaka creates more breeding ground for containers mosquitoes(Gould and Higgs, 2009; Braack *et al*., 2018). In addition, people travelling from other parts of Zambia where seroprevalence and detection of mosquito-borne viruses have been reported to Lusaka could contribute to the spread of the disease in Lusaka. Hence, the study will provide knowledge on the status of arboviruses in selected parts of Lusaka

CHAPTER THREE

3.0. MATERIALS AND METHOD

3.1 Research Design

This study utilized a cross-sectional design and was conducted from February to April 2022, which are rainfall months suitable for mosquito breeding in Lusaka.

3.2 Study Area

Mosquitoes were collected from Kabanana, Kalingalinga, Kamanga, George, Kanyama and the University of Zambia (UNZA) within Lusaka district of Lusaka Province. (Figure 3.1) The sites were selected purposively due to availability of mosquito breeding sites, high human population density, type of housing and informal urban development that support the abundance of mosquitoes.

Lusaka District in Lusaka Province is the capital city, cultural and economic centre of Zambia. Lusaka district is located in the southern half of Zambia. Its central geographical coordinates are 15° 24' 24" S and 28° 17' 13" E. (Simwanda et al., 2020). The approximate spatial extent of Lusaka Province is 21896 Km^2 with eight districts, namely Luangwa, Kafue, Lusaka, Chongwe, Chilanga, Chirundu, Rufunsa and Shibuyunji (Lusaka Provincial Administration, 2021). Lusaka Province, with a population of 2,191,225 the largest among the 10 provinces of Zambia according to the 2010 census (*Zambia 2010 census of population and housing national analytical report*, 2012). Lusaka Province lies within the middle veld of Zambia whose altitude above sea level is between 900 m and 1200 m and is drained by four main rivers, the Kafue, Chongwe, Lunsemfwa and Luangwa. The total mean annual rainfall in the districts is between 800 mm and 1000 mm as they lie in the group II of the agroecological region (Zulu, 2018). Primarily due to its high altitude, Lusaka features a humid subtropical climate according to Köppen climate classification. Its coldest month July has a monthly mean temperature of 14.9 °C (58.8 °F). Lusaka features hot summers and cool winters, with cold conditions mainly restricted to nights in June and July. The hottest month is October, which sees daily average high temperatures at around 32 °C (90 °F). There are three main seasons: a raining warm season between November to March, a dry winter between April and August, and a hot summer from September and October (Zulu, 2018).

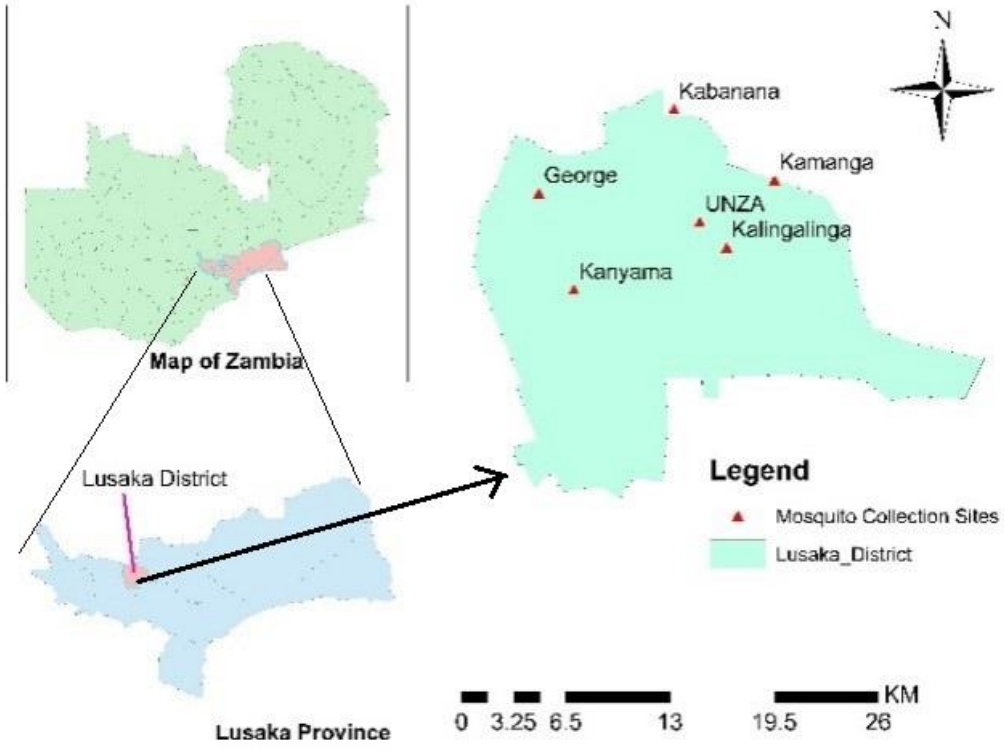

Figure 3.1: Study Site Map (Source: Generated from Arc GIS software)

3.3 Mosquito sampling

In each site, mosquitoes were collected for 3-5 days from 3-6 selected houses approximately 100 m apart from each other from February to April, 2022. A systematic sampling method was applied for selecting study houses in each targeted site. The nearest house from each point was selected within the radius. Another house was selected in cases where the household head or guardian was not willing to participate in the study.

Inclusion Criteria: Houses having families of 4 or more persons were included in the study.

Exclusion Criteria: Houses without occupants or with less than 4 people were excluded from the study

3.4. Sampling Technique

The CDC light traps (John W. Hock Co., Gainesville, FL, USA) with Carbon (IV) oxide produced by yeast fermentation was used for the trapping of mosquitoes in the selected houses between 17:00 hrs to 07:00 hrs, the traps were placed both indoors and outdoors. This was to ensure collection of as many mosquitoes as possible because arbovirus transmission is usually maintained at a low level in a mosquito population. If the number

of mosquitoes collected was insufficient, the position of the traps was randomly changed. The position of the traps was depended on the structure of the house; it was suspended at 1.5 m and above and not near any other sources of artificial light. To avoid RNA degradation, the captured mosquitoes were kept alive during transfer to the laboratory (Iwashita et al., 2018).

3.5 Sample Processing

3.5.1 Mosquito Identification

The adult mosquitoes trapped were killed by freezing at -20^{o}C at the Microbiology Laboratory, Department of Disease Control, School of Veterinary Medicine, The University of Zambia and identified using a standard morphological identification keys under a stereo light microscope to the genus level (Rueda, 2004; Kent, 2006, Gunathilaka, 2017; Coetzee, 2020). Female mosquitoes were sorted into pools having a maximum of 40 mosquitoes according to their genera and location of collection, and stored at -80^{o}C before the molecular detection of mosquito-borne viruses.

3.5.2. DNA Extraction

For specimens morphologically identified according to species, DNA was extracted using Qiagen DNA extraction kit (Qiagen, Hilden, Germany); according to the manufacturer's instructions. Briefly, 180 µl and 20 µl of Buffer ATL and Proteinase K respectively were added to 50 µl of homogenized mosquito samples in a 1.5 ml Eppendorf tube. The mixture was mixed by vortexing followed by incubation at 56 ^{o}C for 1-3 hours for complete lysis. Two hundred (200) µl buffer AL was added to the lysed content and vortexed followed by incubation at 70 ^{o}C for 10 min. Protein precipitation was conducted by adding 200 µl of absolute ethanol followed by pulse-vortexing for 15 s. The mixture was transferred to a QIAamp Mini spin column and centrifuge at 6000 x g for 1 minute and the flow-through discarded. The washing of the column twice was done with 500 µl of each of the washing Buffers W1 and W2, by centrifugation at 6000 x g for 1 min and 20000 x g for 3 min respectively. Finally, DNA was eluted by the addition of 200 µl of buffer AE equilibrated at room temperature to the spin column and centrifuged at 6000 x g for 1 min. The DNA extracted was immediately stored at -80 ^{o}C until amplification by PCR.

3.5.3. PCR Reaction for Molecular Detection of Mosquito species

Cytochrome c oxidase I subunit (COX1) primers was amplified from the extracted DNA as a standard DNA barcoding for molecular identification of mosquito species, a

polymerase chain reaction (PCR) was performed to confirm the species using primers targeting the gene as described by Folmer *et al.*(1994) (Table 3.1).The PCR reactions were carried out in a 10 µL volume reaction mix containing One Taq2X Master Mix with Standard buffer, 2.5 mM $MgCl_2$, 0.16 mM dNTPs premixed, 0.04 µM of each 10 µM primer, and 0.75 units of Taq Polymerase, 2.04 µL of DDW, and 2.00 µL of DNA template. The temperature conditions were as follows: an initial denaturation at 95^{o}C for 1 min followed by 45 cycles at 94^{o}C for 30 s , 40◦C for 1 min (annealing), 72^{o}C for 1 min (extension), and final extension at 72^{o}C for 10 min and hold at 4^{o}C .

3.5.2 Screening for mosquito-borne viruses in mosquitoes

3.5.2. 1 Mosquito Processing

Micro Smash MS-100R Cell Disruptor (Tomy Digital Biology Co., Ltd., Tokyo, Japan) was used to homogenize the sample pools with stainless bead. Dulbecco's Modified Eagle Medium (DMEM: 800 µl) was added to each sterile micro beading tube (2 mL) containing sample pool and was homogenized. The homogenates was then centrifuged at 13,000 rpm for 1 min and 140 µL supernatant was used for viral RNA extraction (Chatterjee et al., 2021).

3.5.2.2 RNA Extraction

RNA was extracted using the QIAamp Viral RNA Mini Kit for nucleic acid extraction (Qiagen, Hilden, Germany); this was done according to the manufacturer's instructions. Briefly, 560 µl of lysis buffer containing carrier RNA and 140 µl of homogenized mosquito tissue supernatant was added to a 1.5 ml microcentrifuge tube. The contents were pulse-vortexed for 15 s before incubation at room temperature for 10 min to ensure complete viral particle and cellular lysis. Protein precipitation was conducted by adding 560 µl of absolute ethanol followed by pulse-vortexing for 15s. Precipitates were removed by centrifugation at 13 000 g for 5 min. The supernatant was carefully withdrawn and passed through a silica-gel column, followed by washing of the column twice with 500 µl of each of the washing Buffers AW1 and AW2, respectively. Finally, RNA was eluted by the addition of 60 µl of buffer AVE equilibrated at room temperature to the spin column and centrifuged at 6000 x g for 1 min. The viral RNA extracted was immediately stored at -80^{o}C until amplification by RT-PCR

3.5.2.3 Detection of Mosquito-borne viruses by reverse transcription PCR

The screening of the mosquito-borne viruses (Alphaviruses, Flaviruses and Phleboviruses) was performed using a One Step PrimeScript RT- PCR Kit (Takara, Shiga, Japan) in a 15µl

reaction mix containing 0.6 µl of Takara PrimeScript Enzyme Mix, 7.5 µl of 2X 1-step buffer, 1 µM of each forward and reverse primer, and 1 µl of RNA template. The samples were incubated at 50 ºC for 30 minutes and 94 ºC for 2 minutes, followed by 50 cycles at 94ºC for 30 seconds, annealing for 30 seconds according to the primer set used (Table 3.1) and final extension at 50 ºC for 30 seconds. The number of cycles was modified from 43 cycles to 50 cycles to increase the detection capacity.

3.5.2.4 Visualization of RT-PCR products

The RT-PCR products and PCR were separated by electrophoresis in a 1.5% agarose gel in 0.5 ×Tris–borate–ethylenediaminetetraacetic acid (TBE) buffer (SERVA, Heidelberg, Germany) stained with Gel Red nucleic acid stain (Phenix Research Products, Candler, NC, USA). Each well was loaded with 5 µl of the PCR product. The samples were separated along with a DNA ladder, Promega 6×blue–orange DNA loading dye (Promega, Madison, USA) at 150 V for 30 min. For purification of gel, a Gel Green stain was used in placed of Gel Red and expected band excised under Transiluminator.

Table 3.1: Primers set for Mosquito Identification and Virus Detection

Name	Target gene or protein	Primer	Primer Sequence (5'→3')	PCR product size (bp)	Annealing Temperature	Reference
Pan-Alphaviruses	NSP4	6692 F	CAY-ACR-YTR-TTY-GAY-ATG-TCD-GC	460	52ºC	(Torri *et al*., 2018)
		7152R	GCR-TCD-ATK-ATY-TTB-ACY-TCC-AT			
Pan-Flaviviruses	NS5	Flavi all S	TACAACATGATGGGGAARAGAGARAA	260-270	53ºC	(Patel et al., 2013)
		DEN4 F	TACAACATGATGGGRAAACGTGAGAA			
		Flavi all AS 2	GTGTCCCAGCCNGCKGTGTCATCWGC			
Pan-Phleboviruses	L segment	L-2779F	CARCATGGWGGTYTDAGRGARATCTA	500	52ºC	(Chambaro et al., 2022)
		L-3287R	TGCARKATKCCYTGCATCATHCCWG)			
Mitochondrial cytochrome c oxidase subunit I	Cytochrome c oxidase subunit 1	LCO1490	GGTCAACAATCATAAAGATATTGG	710	40ºC	(Folmer et al., 1994)
		HC02198	TAAACTTCAGGGTGACCAAAAAATCA			

3.5.2.5. PCR Product purification

The amplified expected band size of PCR product were purified using Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instruction. Briefly, following electrophoresis, excise DNA band from gel was placed in a 1.5 ml microcentrifuge tube. Ten (10) µl Membrane Binding Solution was added per 10 mg of gel slice. The mixture was Vortex and incubated at 50–65 °C to completely dissolve the gel. The SV Mini column was inserted into Collection Tube and dissolved gel was transferred to the Mini column assembly and allowed for 1 minute at room temperature. It was then Centrifuge at 16,000 × g for 1 minute. The flow-through was discarded and Mini column was reinserted into the Collection Tube. 700 µl Membrane Wash Solution (ethanol added) was added and centrifuge at 16,000 × g for 1 min. Again the flow-through was discarded and the washing repeated with 500 µl by Centrifuging at 16,000 × g for 5 min. The Collection Tube emptied and the column assembly was centrifuge for 1 minute to ensure complete evaporation of any residual ethanol before elution of the PCR product. The Mini column was carefully transferred to a clean 1.5 ml microcentrifuge tube and 50 µl of nuclease-free water was added to the Mini column. This was incubated for 1 minute at room temperature and centrifuged at 16,000 × g for 1 min. The eluted DNA was stored at DNA at 4 °C or –20 °C prior to sequencing reaction.

3.5.2.6. Cycle Sequencing reaction

Cycle sequencing reaction was done in a10µl reaction volume mix comprising; 1 µl of purified DNA, 0.5µl BigDye™ Terminator v3.1 Ready Reaction Mix, 2.0 µl of 5X Sequencing Buffer, 4.0 µl of Deionized water (RNase/DNase-free) and 0.5µl of primer set. The Cycling Program was as follows: incubation at 96 °C for 1 minutes; denaturation at 96 °C for 10 seconds; annealing at 50 °C for 5 seconds; extension at 60°C for 2 minutes for 30 cycles and hold at 4 ˚C till infinity.

3.5.2.6. Purification of Sequencing reaction

The sequencing reaction was cleaned to remove the Big dye terminator using Agencourt CleanSEQ Dye-Terminator Removal (Beckman Coulter, Inc.) according to the manufacturer's instructions. Briefly, The Agencourt CleanSEQ was mixed thoroughly to ensure no visible bead pellet remains in the bottle. 10µl of the Agencourt CleanSEQ was added to each sample, 85% ethanol was then added to the mixture and mix by vortexing 7 times. The tubes containing the sample was then placed on the Agencourt plate and allowed for 3-5 minutes to allowed beads to attached to the magnet and the clear supernatant was

withdrawn carefully. Another 100 µl of 85% ethanol was added and allowed for 30 seconds and the washing step was repeated and ensuring that all ethanol was removed. The tubes were allowed to air dry for 10 min on/off the Agencourt plate. 40 µl Deionized water (RNase/DNase-free) was added to the air dry tubes and 35 µl of the cleaned product was collected for sequencing after 5 min and stored at 5 °C until sequencing by capillary sequencer. This was performed using SeqStudio Genetic Analyser (Applied Biosystems, Foster City, CA).

3.5.2.7. Sequence analysis

The nucleotide sequences were assembled and edited in GENETYX, version 13 (GENETYX, Tokyo, Japan) using the ATGC software to obtained a consensus sequence for each pair of sequences. The consensus sequences were compared with available sequences using the Basic Local Alignment Search Tool (BLAST. http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the organisms. The consensus nucleotide sequence was further translated to amino acid by GENETYX ver. 13 software (Genetyx Corp.) for protein blast.

3.5.2.8. Multiple sequences alignment and Phylogenetic Tree

Twenty-two (22) other RNA dependent RNA polymerase (RdRp) amino acid reference sequences of bunyaviruses were obtained from Uniprot website used for alignment (https://www.uniprot.org/uniprotkb?query). Multiple sequence alignment of the reference sequences and translated sequence from this study (Sample 6), was achieved using MAFFT version 7.308 (Katoh and Standley, 2013) in Geneious v 9.1.8 (https://www.geneious.com/).

The alignment file was converted to MEGA format and exported to MEGA 7 (Kumar et al., 2016) for construction of the phylogenetic tree. The phylogenies were constructed using and Maximum Likelihood method. The robustness of the resulting branching patterns was tested using the bootstraps method with 1000 replicates.

3.6. Statistical Analysis

The number of captured mosquitoes was entered into MS excel according to sex and location. The data was imported into Statistical Package for Social Sciences version 21 and a descriptive statistic was performed to report the percentages of Captured mosquitoes from the study areas.

3.7. Ethical considerations

The ethical clearance for this study was obtained from the ethics review committee of Excellence in Research Ethics and Science (ERES) Converge Ethics Committee IRB (Ref. No. 2022-Jan-005). Permission to collect mosquitoes from household was sought from the Ministry of Health Zambia (Ref. No. MH/101/23/10). The authorisation to carry out this study was obtained from National Health Research Authority in Zambia (Ref. No. NHRA 00005/10/05/2022). Consent from the owners of the houses/premises was also obtained and the study procedure was explained to the house or premise owner. They were requested to read or have read to them the information sheet, comprehend and sign/ thumbprint an informed consent form prior to sample collection. The supporting document are attached in Appendices (A, B, C, D, E , F,G, and H).

The traps used were set in the sitting rooms and not bed rooms to prevent the disturbing sound from the fan that may hinder sleep. The houses are coded to avoid any information that can be used to trace the house owner and sample pooled based on location not household to avoid traceability of the house owner.

CHAPTER FOUR

4.0. RESULTS

4.1. Captured Mosquitoes from Selected parts of Lusaka District

A total of 2404 mosquitoes were collected from the study locations, comprising of 86.2% females and 13.8% males. The highest number of mosquitoes were collected from UNZA and the least was collected from Kabanana (Table 4.1).

Table 4.1:Captured Mosquitoes from Selected Areas of Lusaka District

Sample Location	Sex of mosquito		Total
	Female	Male	
	N	N	N
	(%)	(%)	(%)
George	314	49	363
	(86.5)	(13.5)	(100.0)
Kabanana	74	3	77
	(96.1)	(3.9)	(100.0)
Kalingalinga	108	14	122
	(88.5)	(11.5)	(100.0)
Kamanga	391	27	418
	(93.5)	(6.5)	(100.0)
Kanyama	185	211	396
	(46.7)	(53.3)	(100.0)
UNZA	1000	28	1028
	(97.3)	(2.7)	(100.0)
Total	2072	332	2404
	(86.2)	(13.8)	(100.0)

4.2. Morphological Identification of Mosquito Genus

The highest captured mosquitoes were the *Culex* genus based on morphological identification (Appendix I) followed by *Aede*s and the least was *Anopheles* both captured only in UNZA (Table 4.2).

Table 4.2: Genera of mosquitoes captured in selected areas of Lusaka District

Genus	George	Kabanana	Kalingalinga	Kamanga	Kanyama	UNZA	Total
Aedes	0	0	0	0	0	2	2
Anopheles	0	0	0	0	0	1	1
Culex	314	74	108	391	185	997	2069
Total	314	74	108	391	185	1000	2072

4.3. DNA Barcoding for Mosquito Identification

The amplified product for molecular characterization of the identified Mosquitoes genera confirmed the species in pool sample 4, 5 and 6 are *Aedes aegypti*, *Anopheles rufipes,* and *Culex quinquefasciatus* respectively (Figure 4.2). The *Aedes* and *Anopheles* mosquitoes were collected from the University of Zambia while the *Culex* mosquito was collected in Kanyama.

The blast result of the obtained sequences (Appendix J) from NCBI website for sample 4 showed a 78.11% identity with *Aedes aegyti*, Sample 5 Showed a 99.80% identity with *Anopheles rufipes* and Sample 6 showed a 92.69% identity with *Culex quinquefasciatus* (Table 4.3)

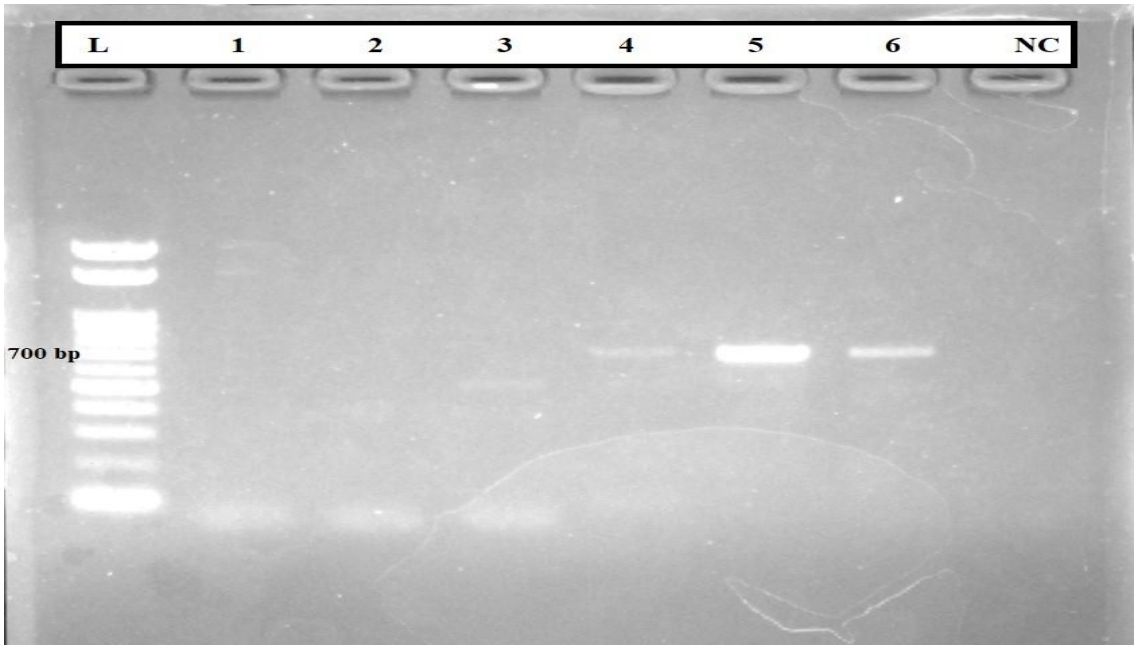

Figure 4.1:Mosquito Identification Using Mitochondrial Cytochrome Oxidase Subunit 1. PCR product (The Cytochrome oxidase c subunit 1 gene) size of approximately 700 base pairs obtained on sample 4,5, 6. L, NC and lane numbers 1 - 6 correspond to ladder, negative control (containing the PCR reaction mix with sample) and represented pooled samples respectively**.**

Table 4.3: Molecular Species Identification of Mosquitoes

Mosquito Genus	Species Name	Closely Related (Accession No.)	Blastn		
			Identity (%)	Query Cover (%)	E-Value
Aedes	*Aedes aegypti*	*Aedes aegypti* (KX446467.1)	78.11	82	1e-51
Anopheles	*Anopheles rufipes*	*Anopheles rufipes* (LC473604.1)	99.8	97	0.0
Culex	*Culex quinquefasciatus*	*Culex quinquefasciatus* (KF407080.1)	92.69	90	0.0

4.4. Mosquito-borne viruses Screening from Mosquito Pools

Three (3) out of the seventy-one (71) pooled samples tested produced bands of expected size of approximately 500 base pairs for Pan Phlebovirus virus screening. However, only the strongly positive sample (Sample 6) from Kanyama was sequenced successfully (Figure 4.3) All the seventy-one (71) pooled of mosquitoes tested were negative for both pan Flaviviruses and Alphaviruses screening.

4.5. Molecular Identification of Culex Bunyavirus 2

The blast result from NCBI websites of the consensus nucleotide sequence (Appendix J) obtained from the strong positive sample (sample ID:6) (Figure 4.3) showed 85.69% identity with Culex bunyavirus 2 from the Genbank with an accession number:MW434616.1, E-value:8.00E-161, and Query cover: 99% . The amino acid sequence blast result showed 97.45% identity with Culex bunyavirus 2 from the Genbank with an accession number:QRW41991.1, E-Value: 3.00E-99, and Query cover:94%.

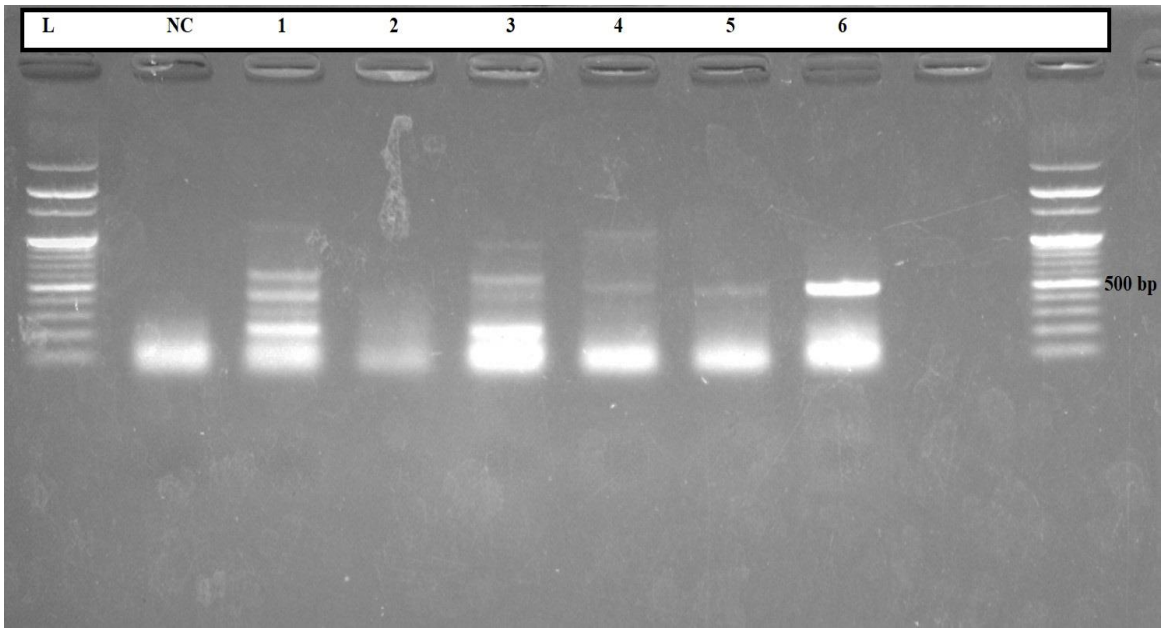

Figure 4.2: Detection of L segment of Phleboviruses PCR product (partial segment of RdRp of phleboviruses) size of 500 base pairs was obtained on sample 6. L, NC and lane number 1 - 6 correspond to ladder, negative control (RT-PCR reaction mix with the sample) and represented pooled samples. The ladder size was 100 bp and 1.5% Agarose gel. Sample 1-4 produces unexpected band sizes as seen on the gel**.**

4.6. Phylogenetic Analysis

Phylogenetic tree analysis segregated the sequences according to their respective genus based on the RdRp protein sequences. The sequence in this study marked in red clusters with *Culex bunyavirus 2* and *Culex bunya-like virus* which are unclassified bunyaviruses (Figure 4.3).

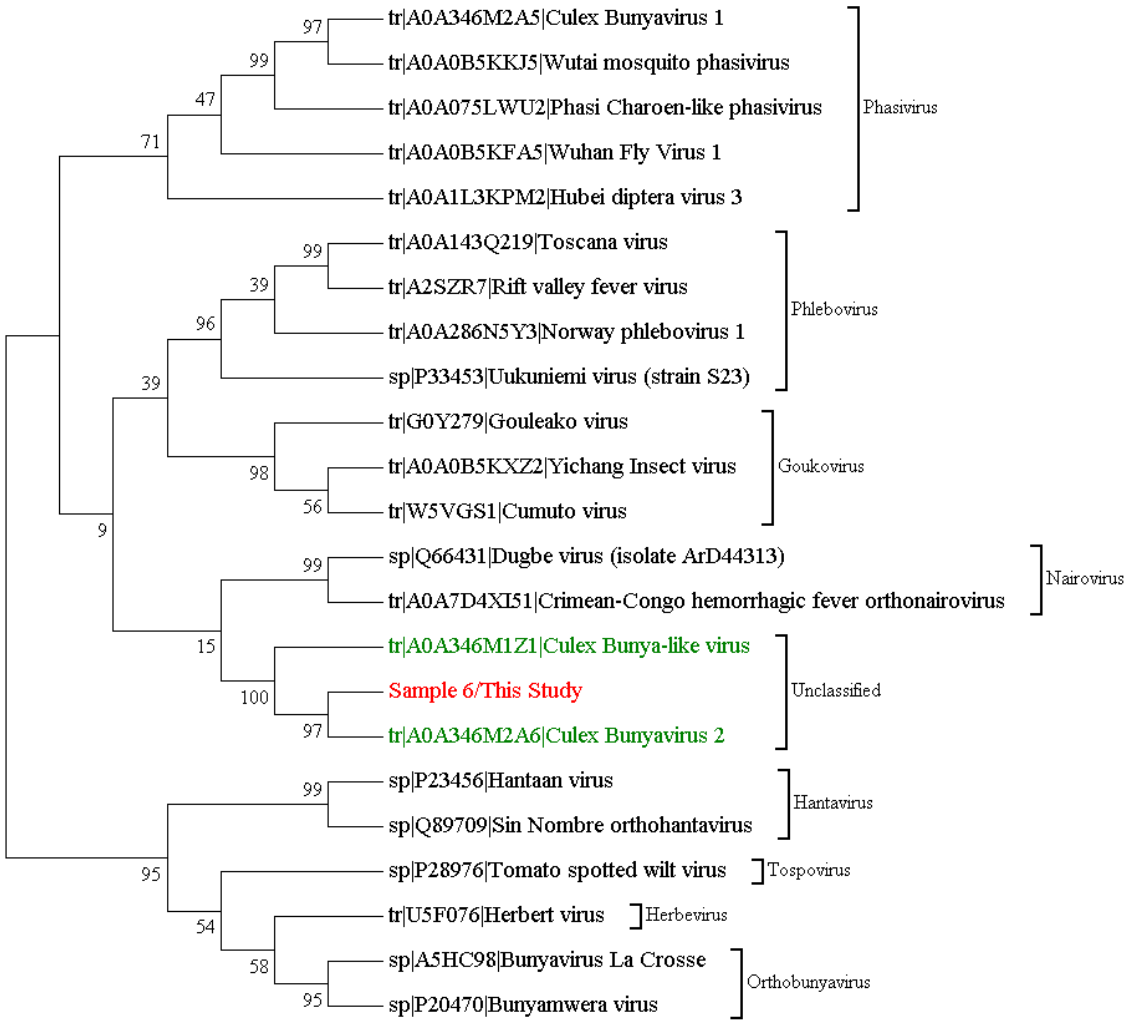

Figure 4.3: Phylogenetic Analysis of Culex bunyavirus 2 in Zambia and other Bunyaviruses based on the RdRp gene. The evolutionary history was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model. The tree with the highest log likelihood (-5519.52) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The analysis involved 23 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 135 positions in the final dataset. Evolutionary analyses were conducted in MEGA7

CHAPTER FIVE

5.0. DISCUSSION

This study was carried out to identify the captured mosquito vectors circulating in selected parts of Lusaka District and determine the viruses circulating in the mosquitoes with possibility of being transmitted to humans in the communities of Lusaka district, Zambia. In this study mosquitoes were collected between February and April 2022, 2404 mosquitoes were collected from six sites within the District, comprising 2072 females and 332 males.

An overall of 86.2 % population of mosquitoes were females, this could be attributed to the preference of females coming close to human habitation to obtain their blood meal for development of their eggs (Harbach, 2007). The percentage of females were higher across the sites except for Kanyama where the males accounted for 53.3 % and females 46.7 %. This could be attributed to the presence of stagnant water bodies serving as breeding grounds in close proximity with the people. Kanyama is characterized by flooding and high human population density due to its proximity to the central business district of Lusaka. On the other hand, the presence of large water bodies at the University of Zambia could explain why the highest number of mosquitoes and the presence *Aedes aegypti* and *Anopheles rufipes* which were captured from the institution as water bodies are breeding grounds for mosquitoes.

The mosquito genera and species identified have been previously reported in Lusaka and other parts of Zambia (Masaninga et al., 2012; Masaninga et al., 2014b; Wastika et al., 2020). Consistent with previous studies, *Culex* spp. constituted the most abundant mosquito genus (Masaninga et al., 2012; Torii et al., 2018).This finding is in agreement with the established fact that Southern household mosquitoes, precisely *Culex quinquefasciatus*, which is the dominant species in this study are mostly dominant in tropical and sub-tropical urban environments like Lusaka where environmental conditions tend to favour their propagation (Soh and Aik, 2021). Another interesting finding from this study was the identification of one adult *Anopheles* mosquitoes in the urban setting of Lusaka where *Anopheles* mosquitoes are perceived to be absent. Only one study has reported *Anopheles* larvae in peri-urban setting of Lusaka District with no adult anopheline collection (Masaninga *et al*, 2012). Hence the need to embark on increased mosquito surveillance in Lusaka to ascertain the vector composition. The lack of *Anopheles* could be

a result of increased city extension that could significantly destroyed mosquito breeding sites for *Anopheles* mosquitoes. This study further identified Aedes species in low numbers, this could be attributed to use of CDC light traps in this study. Since CDC light trap mostly capture the night biting mosquitoes, it is possible that day biting species, such as many *Aedes* spp., may have been underrepresented

Despite *Anopheles* and *Aedes* were in very low numbers.in this study It is important to pay keen attention to vector surveillance as the transmission of mosquito-borne arboviruses is reliant on the presence of these vectors. The assessments of the risk of mosquito-virus emergence to a new region or maintenance of arboviruses within a particular region depends on the knowledge of the distribution and competence of these mosquitoes (Schulz and Becker, 2018). On the other hand, the high abundance of *Culex* could be explained to be as a result of poor drainage and sewage system, intensified by the uncontrolled population growth in suburban areas of Lusaka, such as George, Kamanga, Kalingalinga, Kanyama, Kabanana, as well as the University of Zambia hostels where samples were collected.

Culicine mosquitoes are generally associated with polluted waters with high organic matter. This could explain the abundance of *Culex* spp in this area where there is no proper waste collection and disposal or sewage drainage system, increasing the extent of pollution and providing a suitable breeding ground for mosquitoes. These findings are in agreement to studies carried out in Kinshasa, Democratic Republic of Congo (Mbanzulu et al., 2017), in Kyela district of Tanzania (Bisimwa, *et al.*, 2016) and in Yaounde town of Cameroon (Nchoutpouen et al., 2019) that correlate the presence of *Culex* mosquitoes to the polluted environment which is an evidence of urbanization and unplanned settlements.

In this study, no known human pathogenic Mosquito-borne virus was detected in all the 71 pools screened. Three positive pools were recorded for Pan-phlebovirus virus screening but only 1 was sequenced as a result of poor band appearance and the detected virus was Culex bunyavirus 2. To the best of our knowledge, this was the first study that attempted to unravel mosquito-borne viruses circulating in mosquitoes from the urban setting in Lusaka district of Zambia which serves as the capital city of Zambia. In this study for the first time in Zambia, the pan-phlebo reverse transcription-PCR assay detected a partial genomic sequence of Culex bunyavirus 2. The RNA directed RNA polymerase gene (RdRp) L segment which had 85.67% homology score after blast analysis from the NCBI website with a Culex bunyavirus 2 detected in California USA from a metagenomics

analysis of *Culex* mosquito virome (Altan et al., 2018). The amino acid sequence blast result showed 97.45% identity with Culex Bunyavirus 2 making our detected virus most likely a Culex bunyavirus 2

The phylogenetic analysis on the partial RdRp sequence in this study characterized the detected virus as an unclassified bunyavirus that formed a separate clade from other classified bunyaviruses with Culex bunyavirus 2 and Culex bunya-like virus. These viruses have been inferred to belong to the *Phenuiviridae* family (Faizah et al., 2020). Although the viruses are yet to be assigned a genus in the Bunyaviruses group (Schoch et al., 2020)and the biology of the viruses is yet to be established.

Not much has been described about this novel virus. The virus may possibly be an insect specific virus like the sister clade Goukovirus genus, which includes the agent Guoleako virus, Cumuto virus and Yinchang insect viruses which are regarded as insect specific viruses (Carvalho and Long, 2021) and the phasivirus genus. The virus was similar with several other mosquito-associated *Bunyaviridae* that have been found using metagenomics approach in the United State of America and in Japan (Altan et al., 2018; Chandler et al., 2015; Faizah et al., 2020).

The result in this study was based on partial sequences of the L segment (RdRp) approximately 500bp of bunyaviruses directly detected from mosquitoes. However, other regions of bunyaviruses nucleotide sequences (such as the S and M segment) were not assayed, and there is a probability that these sequences may differ. Therefore, more sequence information will be needed to categorically establish the detailed taxonomic status of this novel mosquito-borne virus. It is also important to further study this virus as proper identification and characterization of viruses in Mosquitoes may provide the evolutionary history viruses (Hameed *et al.*, 2021).

As a result of the above limitation in this study, we do not know if the detected virus belongs to the insect-specific viruses where its sister clade (Guokovirus, Phasivirus) belongs and has the ability to only infect arthropods but does not replicate in mammalian cells or whether it can cause diseases like other phleboviruses that cause diseases such as the Rift valley fever virus. Hence, the need to further investigate this virus as the detection process differs from others that have been detected via metagenomics Next generation sequencing (Chandler, Liu and Bennett, 2015; Hobson-Peters *et al.*, 2016; Altan *et al.*, 2018; Hameed *et al.*, 2021).

Further studies of this virus will be necessary to ascertain whether it is an Insect specific virus, since some ISVs have shown the ability to temper with arboviruses replication and vector competence of mosquitoes making them promising agents as biological control, vaccines candidates, and diagnostic platforms for arbovirus diseases (Carvalho and Long, 2021).

Currently, there is little or no information about these unclassified bunyaviruses and their evolution in mosquitoes. The discovery and characterization of these non-classified viruses can provide valuable information on their genetic diversity, ecology, evolution, and most importantly, the potential to threaten animal and human health (Hameed et al., 2021b). This finding supported the existence of this virus in mosquito populations and the need for further investigation is necessary due to the astonishing mutation potential of viruses that may lead to the emergence of new vertebrate diseases. Continuous surveillance is therefore required to prevent possible future outbreaks of mosquito-borne viral diseases of either man or animals.

It is important to note the limitations encountered during this study so that the results are not extrapolated beyond the study population. This current study did not cover a year cycle to estimate the seasonality of mosquito abundance and composition since it has been shown that mosquito abundance and composition depends on seasons (Stoek et al., 2022). The sampling was done for only a short period between February and April during the rainy season in Lusaka. Trapping method and time for collection were also limiting factors in this study. The CDC light trap is not suitable for capturing *Aedes* mosquitoes and also most of the mosquitoes collected were from indoors due to security concerns of setting traps outdoors.

The absence of known pathogenic mosquito-borne viruses among the assayed mosquitoes could be due to small sample size as mosquitoes borne viruses are usually circulating in low numbers in their vector(Iwashita et al., 2018). Hence the need for an increased surveillance or larger studies to provide a true prevalence of arboviruses circulating in Lusaka District. Furthermore, the use of traps suitable for *Aedes* collection such as the BG sentinel traps known for increased capturing of *Aedes* mosquitoes as they are the vectors for most arboviruses causing disease in humans (Mbanzulu et al., 2017), will have to be employed.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1. Conclusion

Mosquitoes from six selected areas of Lusaka District were collected and analysed in this study with the objectives to identify the mosquitoes, detect and phylogenetically characterize mosquito-borne viruses circulating among the mosquitoes in selected parts of Lusaka district. In this study three genera of mosquitoes known to transmit arboviruses were identified to be in circulation in selected parts of Lusaka District namely, *Aedes*, *Anopheles*, and *Culex* mosquitoes. Molecular detection of not fully identified and characterized unclassified bunyavirus, a mosquito-borne virus genetically related to Culex bunyavirus 2 in one of the pools tested from Kanyama township indicates the presence of this virus circulation in mosquitoes in selected parts of Lusaka District.

6.2. Recommendations

The following recommendations are made

i. Better Mosquito trapping tools and collection in other parts of Lusaka throughout the year is necessary to establish the presence of Culex bunyavirus 2 and other mosquito-borne viruses in Zambia
ii. A Metagenomics analysis or Whole genome sequencing and viral culture to properly characterized the Culex bunyavirus 2 and its potential to cause disease in humans or animals should be determined

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APPENDICES

Appendix A: Information sheet

No……

Dear Participant,

You are invited to participate in a study titled "INVESTIGATION OF MOSQUITO (Diptera: Culicidae) -BORNE VIRUSES CIRCULATING IN SOME SELECTED AREAS OF LUSAKA DISTRICT, ZAMBIA".

My name is Yusuf Eshimutu Abu, the principal investigator in this study from the school of Veterinary Medicine at the University of Zambia. This study is in partial fulfilment of the requirement for a Master degree in Tropical Infectious Diseases and Zoonosis. This study aimed to detect medically important mosquito-borne viruses circulating in some selected areas of Lusaka District in mosquitoes

The study will require the collection of mosquitoes at your house or premises both indoors and outdoors for 3days consecutively per month for 3 months using traps. CDC light traps and BG sentinel traps will be set both indoors and outdoors randomly. This is to ensure the collection of as many mosquitoes as possible because arbovirus transmission is usually maintained at a low level in the mosquito population. If the number of mosquitoes collected was insufficient, the position of the traps or type of traps will be randomly changed. The position of the traps will be depended on the structure of the house. CDC light traps will be suspended 1.5 m above the ground inside and outside of the houses but not near any other sources of artificial light and BG sentinel traps will be placed in the house with enough space or outside of the house. CDC light traps will be operated from dusk to dawn and BG sentinel trap will be operated for 3 days continuously.

There is no risk attached to the traps but the participant may try to cope with the noise from the trap fan and light as an attractant to the mosquitoes. Participation in this survey is voluntary and you have the right to withdraw. There is no monetary compensation as regards this study but have the researcher's thanks for your participation. The participation is anonymous, and no one will be able to link your house or premises to you. Any report

of this research that is made public will not include your name or any individual information by which you could be identified.

In case you have any questions about this study please do not hesitate to contact the student researcher, Yusuf Eshimutu Abu - vet2100098@student.unza.zm or

The Supervisor,
Dr. Tapiwa Lundu,
Department of Biomedical Sciences,
School of Veterinary Medicine.
The University of Zambia
P.O. Box 32379, Lusaka,
ZAMBIA.
Mobile: +260-973-363-449
OR

ERES Converge,
Plot No. 272, Cnr Olive Tree Meanwood Road,
Meanwood Ibex,
Lusaka
Tel: +260955155633 or +260955155634
Cell: +260977493220
Email: eresconverge@yahoo.co.uk

Appendix B: Consent Form

By undersigning this consent form, you agree and declare that;

You have been informed about this study's purpose, procedures, possible benefits, and risks.

You have been given the chance to ask questions before you sign.

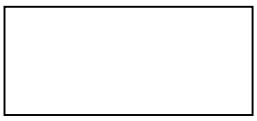
You have voluntarily agreed to participate in this study.

Please indicate Yes or No

I agree to allow my house/premises to participate in this study

☐Yes ☐No

Name of participant: _____



_____ _____

Signature of participant or Thumb print Date

In signing here, I agree that I have read and understood the agreement/consent form and agree to participate in the study.

Name of Witness:

_____

_____ _____

Signature of Witness Date

_____ _____

Signature of Investigator Date

Appendix C: Information sheet in Nyanja

PEPALA YA CHIZIWISO

NUMBALA …….

Wotengako Mbali,

Mukuitanidwa kuti mutengeko mbali pa Lisechi (research) yochedwa "KUFUFUZA PA ZA UZUZU (mosquito) NDI MA MATENDA YAMENE YA MAPEZEKA MU UZUZU MUDELA YA LUSAKA DISTRICT MUNO MU ZAMBIA".

Dzina langa ndine Yusuf Eshimutu Abu, wofufuza wamkulu mu lisechi iyi ndipo ndichokela kusukulu ya Veterinary Medicine pa University of Zambia. Lisechi iyi ndiyamamphuzilo ya digili (degree) ya Masters mu matenda yochedwa Tropical Infectious Diseases and Zoonosis. Iyi lisechi yisakila matenda yobwela na tulombo tochedwa viruses tomwe tupezeka mu uzuzu, chifukwa tifuna kuziwa kuti kapena tulimo muno muLusaka district.

Lisechi iyi izafuna ku gwira uzuzu mkati mwanyumba yani ndi pabwalo kukwanila masiku yatatu – 3-days pa mwezi mbaka myezi yitatu. Pogwila uzuzu, tizasebenzesa ma CDC trap (misampha) ndi BG trap. Tikatelo, tizagwila uzuzu wambiri kuti tiwone ngati ma viruses yalimo mu uzuzu. Nthawi zambili, ma viruses yapezeka yan'gono maningi mu mosquito, ndiye nicholinga kugwila mosquito yambili. Ngati nambala ya mosquito ili kugwilidwa ndiyayin'gono, tizachinja poika ma trap, kulingana ndi plani yanyumba. Ma CDC trap yazaikidwa pa mwamba ndipo mamita 1.5m kucokela pansi. Ma CDC trap yazaikidwa mkati ndi pabwalo pa nyumba yanu. Ma BG trap nayonso yazaikidwa mkati olo pabwalo pa nyumba yanu. Ma trap yali ndi malaiti yabo ndipo sikuzafunika malaiti yenanso pafupi. Ma CDC trap yazasebenza kuchokela mumazulo mbaka mumawa. Ma BG traps yazasebenza nthawi yonse kufikila masiku yatatu.

Ma trap (misampha), ilibe chiwopsezo (danger or risk) kubantu, koma kuli phokoso yayin'gono olo chongo cocokela ku fani ya ma trap, ndipo light nayonso izankhala yoyaka kuti yiyitane mosquito kungena mu trap. Sitikakamiza munthu aliyense kutengako mbali mu lisechi iyi, ndipo mungakane tutengako mbali nthawi iliyonse. Kulibe ndalama zolipila anthu omwe azatenga mbali mu lisechi iyi. Mazina ndimalo yabonse wotengako mbali mu lisechi iyi siyazatengeka, ndipo zonse zizankhala zachisinsi. Kulibe azaziwa kuti munatengako mbali mu lisechi iyi.

Ngati muli ndi mafunso okhuza liseci (research) iyi, munga funse mafunso kwa a Yusuf Eshimutu Abu - vet2100098@student.unza.zm

Kapena mtsogoleri:

Dr. Tapiwa Lundu,
Dipatimenti ya Biomedical Sciences,
Sukulu ya Veterinary Medicine.
University of Zambia
P.O. Box 32379, Lusaka,
ZAMBIA.
Mobile: +260-973-363-449

KAPENA
ERES Converge,
Plot No. 272, Cnr Olive Tree Meanwood Road,
Meanwood Ibex,
Lusaka
Tel: +260955155633 kapena +260955155634
Selo: +260977493220
Imelo: eresconverge@yahoo.co.uk

Appendix D: Concept form in Nyanja

FOMU YOVOMEREZA

Posaina pansi pa fomu yololeza iyi, mukuvomera ndikulengeza kuti;

Mwadziwitsidwa za cholinga cha lliseci (research) iyi, ndondomeko, ubwino ndi zoopsa zomwe zingatheke.

Mwapatsidwa mupata wofunsa mafunso musanasaine.

Mwavomera mwakufuna kwanu kutengako nawo mbali mu liseci iyi.

Pulizi onetsani Inde kapena Ayi

Ndikuvomera kulowa m'nyumba/malo anga kuchita liseci.

☐Inde ☐Ayi

Dzina la otenga nawo mbali: _____

_____ _____ _____

Siginecha ya otenga nawo mbali kapena Chala chachikulu Tsiku

Posaina apa, ndikuvomereza kuti ndawerenga ndikumvetsetsa fomu ya mgwirizano/chivomerezo ndipo ndikuvomera kutenga nawo gawo mu liseci.

Dzina la Mboni: _____

_____ _____

Siginecha la Umboni Tsiku

_____ _____

Siginecha la Wofufuza Tsiku

Appendix E. Institutional Approval

THE UNIVERSITY OF ZAMBIA
SCHOOL OF VETERINARY MEDICINE
OFFICE OF THE ASSISTANT DEAN (POSTGRADUATE)

Telephone: 293727
Telegrams: UNZA LUSAKA
Telex: UNZALU ZA 44370
Fax: 293727/253952
School Fax: 293727
Vet. Clinic Telephone: 291515

P.O. Box 32379
Lusaka, Zambia

Your Ref:

Our Ref:

11th January, 2022

Yusuf Eshimutu
Department of Biomedical Studies
School of Veterinary Medicine
University of Zambia
P.O. Box 32379
LUSAKA

Dear Yusuf,

RE: APPROVAL OF RESEARCH PROPOSAL

At the meeting of the School Board of Graduate Studies held on 10th January 2022, your research proposal entitled ***'An investigation of mosquito (diptera: culidae) borne viruses circulating in Lusaka Zambia.'*** was tabled and discussed. I am therefore pleased to inform you that the research proposal was subsequently approved by the Board.

On behalf of the Board, I wish you success as you apply for ethical approval and carry on with you research activities.

Yours sincerely

Dr Chisoni Mumba
ACTING ASSISTANT DEAN (PG), SCHOOL OF VETERINARY MEDICINE

Cc *Dean, School of Veterinary Medicine*
Head, Biomedical Studies
File

Appendix F: Ethical Approval

Plot No. 272, Cnr Olive Tree Meanwood Road,
Meanwood Ibex
Lusaka - Zambia
Tel. +260 955 155 633
+260 955 155 634
Cell: +260 977 493 220
Email: eresconverge@yahoo.co.uk

I.R.B. No. 00005948
F.W.A. No 00011697

3rd February, 2022.

Ref. No. 2022-Jan-005

The Principal Investigator
Mr. Yusuf Eshimutu Abu
Department of Biomedical Sciences
School Of Veterinary Medicine
The University of Zambia
P.O. Box 32379
Lusaka, Zambia

Dear Mr. Abu

RE: INVESTIGATION OF MOSQUTIO (Diptera: Culicidae) BORNE VIRUSES CIRCULATING IN SOME SELECTED AREAS OF LUSAKA, DISTRICT, ZAMBIA

Reference is made to your protocol submission. The IRB resolved to approve this study and your participation as Principal Investigator for a period of one year.

Review Type	**Ordinary**	Approval No. **2022-Jan-006**
Approval and Expiry Date	Approval Date: 3rd February 2022	Expiry Date: 2nd February, 2023
Protocol Version and Date	Version - Nil.	2nd February, 2023
Information Sheet, Consent Forms and Dates	• English.	2nd February, 2023
Consent form ID and Date	Version - Nil	2nd February, 2023
Recruitment Materials	Nil	2nd February, 2023
Other Study Documents	Data Collection Sheet, Focus Group Discussion.	2nd February, 2023
Number of participants approved for study	-	2nd February, 2023

Specific conditions will apply to this approval. As Principal Investigator it is your responsibility to ensure that the contents of this letter are adhered to. If these are not adhered to, the approval may be suspended. Should the study be suspended, study sponsors and other regulatory authorities will be informed.

Conditions of Approval

- No participant may be involved in any study procedure prior to the study approval or after the expiration date.
- All unanticipated or Serious Adverse Events (SAEs) must be reported to the IRB within 5 days.
- All protocol modifications must be IRB approved prior to implementation unless they are intended to reduce risk (but must still be reported for approval). Modifications will include any change of investigator/s or site address.
- All protocol deviations must be reported to the IRB within 5 working days.
- All recruitment materials must be approved by the IRB prior to being used.
- Principal investigators are responsible for initiating Continuing Review proceedings. Documents must be received by the IRB at least 30 days before the expiry date. This is for the purpose of facilitating the review process. Any documents received less than 30 days before expiry will be labelled "late submissions" and will incur a penalty.
- Every 6 (six) months a progress report form supplied by ERES IRB must be filled in and submitted to us.
- A reprint of this letter shall be done at a fee.

Should you have any questions regarding anything indicated in this letter, please do not hesitate to get in touch with us at the above indicated address.

On behalf of ERES Converge IRB, we would like to wish you all the success as you carry out your study.

Yours faithfully,
ERES CONVERGE IRB

Dr. Jason Mwanza
Dip. Clin. Med. Sc., BA., M.Sc., PhD
CHAIRPERSON

Appendix G: Permission of study by Ministry of Health, Zambia

All Correspondence should be addressed to the
Permanent Secretary
Telephone: +260 211 253030/5
Fax: +260 211 253344

REPUBLIC OF ZAMBIA
MINISTRY OF HEALTH

In reply please quote:
MH/101/23/10
No.....

NDEKE HOUSE
P. O. BOX 30205
LUSAKA

15 February, 2022

Mr. Yusuf Eshimutu Abu
University of Zambia
Department of Biomedical Sciences
School of Veterinary Medicine
Box 32379
LUSAKA

RE: PERMISSION TO CONDUCT RESEARCH

The Ministry of Health is in receipt of your request to collect mosquito samples from households in Lusaka in research entitled **"Investigation of Mosquito borne viruses circulating in some selected areas of Lusaka District, Zambia".**

I wish to inform you that permission to conduct Research has been granted and information obtained will be used only for the intended purpose as stipulated in the request.

By copy of this letter, Provincial and District Health Directors are hereby informed.

Prof. Lackson Kasonka
Permanent Secretary- (TS)
MINISTRY OF HEALTH

Cc: PHD- Lusaka Province
Cc: DHD- Lusaka District

Appendix H: Approval to conduct Research by NHRA

NATIONAL HEALTH RESEARCH AUTHORITY
Paediatric Centre of Excellence, University Teaching Hospital, P.O. Box 30075, LUSAKA
Chalala Office Lot No. 18961/M, Off Kasama Road, P.O. Box 30075, LUSAKA
Tell: +260211 250309 | Email: znhrasec@nhra.org.zm | www.nhra.org.zm

Ref No: NHRA00005/10/05/2022 **Date: 10th May, 2022**

The Principal Investigator,
Yusuf Eshimutu Abu
University of Zambia
Lusaka, Zambia.

Dear Yusuf Eshimutu Abu,

Re: Request for Authority to Conduct Research

The National Health Research Authority is in receipt of your request for authority to conduct research titled **"Investigation of Mosquito (Diptera: Culicidae) -Borne Viruses circulating in Some Selected Areas of Lusaka District, Zambia."**

I wish to inform you that following submission of your request to the Authority, our review of the same and in view of the ethical clearance, this study has been **approved** on condition that:

1. The relevant Provincial and District Medical Officers where the study is being conducted are fully appraised;
2. Progress updates are provided to NHRA quarterly from the date of commencement of the study;
3. The final study report is cleared by the NHRA before any publication or dissemination within or outside the country;
4. After clearance for publication or dissemination by the NHRA, the final study report is shared with all relevant Provincial and District Directors of Health where the study was being conducted, University leadership, and all key respondents.

Yours sincerely,

Prof. Godfrey Biemba
Director/CEO
National Health Research Authority

Appendix I: Pictorial Presentation of Mosquitoes Collected

***Aedes aegypti* from UNZA**

Males Mosquito

***Culex tigripes* from George Compound**

***Anopheles rufipes* from UNZA**

Ventral View of *Culex quinquefasciatus*

Side View of *Culex quinquefasciatus*

Appendix J: Nucleotide Sequences

1. Culex bunyavirus 2 RNA dependent RNA Polymerase partial sequence

>Sample 6: Nucleotide sequence of Culex Bunyavirus 2 detected from Culex quinquefasciatus in Kanyama, Zambia (501)

GGAAATTTATGGGCTGACTATCAAGTCAGGTTGCTAGCCCTGCTAGTTGA
AACGTACTCGAGGACTCTTTGCAAGGAATTTGACTGTGAGGCCATGACCC
ATCCTGATCAGAAACTAGAGGTTGTGGAGCGCCATAAGTCCATGGTGAA
GAGGATAATGCATGCTACTGGTCGGAAGGCGTCTGAGTACCAATGCTCA
GCTGACAAAAAGAGTTGGAACAACAACCTGGTGATGCCTGCCTTATCTAT
ACCCCTTCTGATGCTTCTTCCTAAGAAGATGCACGGGACTGTTCAGCGAG
TCCTGAATTTGTGGAACGAGAGACTAGTGAAATTACCTCATGGCGTCATG
AAATTGCTGGTGGCCGGCGTAGAGTTATCTGACCCAACCTACAAGACACT
TATGGAGGAGTTTGAAGACCCTGGATGCTTCAATGGAGAGCCTCTCCTTC
CCAATCCTGGCTCAGCCTTCTGCATTCTCCGGCCTGGAATGATGCAAGGA
AAT

>Sample 6: Translated Amino acid sequences (167)

GNLWADYQVRLLALLVETYSRTLCKEFDCEAMTHPDQKLEVVERHKSMVK
RIMHATGRKASEYQCSADKKSWNNNLVMPALSIPLLMLLPKKMHGTVQRV
LNLWNERLVKLPHGVMKLLVAGVELSDPTYKTLMEEFEDPGCFNGEPLLPN
PGSAFCILRPGMMQGN

2. ***Aedes aegyti*** **Mitochondrial Cytochrome oxidase c subunit 1 sequences**

> Nucleotide sequences of Aedes aegypti detected in UNZA (425)

TCCACGAAGGGCCCCTTGGCGGGAGGAGGTATGAGGGTTGACCCCTCTA
AAAATTAGAATGGGCCCATGTACGACGGAGGAAAAACAATAATAAATAG
CTGTAATAACTACAAGCCAAACAATAATTGTACGATCAAAGTAATCCCTG
ACAAACGTATATTAAGAACAGTTGTAATAKATTTACTGCCCCTAAATTGA
GGAGTTCCAGCTAGTGAAAGAAAAAAGTAAACAACAGAACTCCSGATGA
CTGTCCTGAGAGRAGGGATAACTGTTCACCAGTTCTGCTCCTTTTCTCTAT
GATTTGAGATAGAAATCATGAAGGAGTAGATTCAAAASTTATTTATTCAT
GCAAGAACTGTATCAGKRTCCTAGTATTAAGGACTATCAATTGGATCCTC
CAATTATATTGCATTACTATAAGAAAT

3. ***Anopheles rufipes*Mitochondrial Cytochrome oxidase c subunit 1 sequences**

>Nucleotide sequences of *Anopheles rufipes* detected in UNZA (704)

AAATTAAAGGKTGGKTTTGGGTMATWAAATAGGAKCCCCTCCTCCCGCA
GGSYTCTWAAAGAAGGATGTATTTAAATTTCGATCAGTAAGAAGTATAG
TAATAGCTCCGGCTAGTACTGGTAAAGATAGTAATAATAAAATTGCTGTA
ATTACAACTGATCAAACAAATAATGGTATTCGGTCAAGAGTGATTCCAGG
AGATCGTATATTAATAACTGTTGTAATAAAATTTACTGCTCCTAAAATTG
ATGAAATTCCTGCTAAGTGTAAAGAAAAAATAGCTAAATCAACTGAAGC
TCCGGCATGAGCAATCCCCGATGAAAGAGGAGGATATACAGTTCAACCT
GTTCCTGCTCCATTTTCTACTATACTTCTAGAAATAAGTAACGTTAATGAA
GGGGGAAGTATTCAAAATCTTATATTATTTATTCGAGGGAATGCTATGTC
AGGAGCTCCTAATATTAAAGGAACTAATCAATTTCCAAATCCTCCAATTA
TAATAGGTATTACTATAAAAAAAATTATAATAAAAGCATGAGCAGTAAC
AATAACATTATAAATTTGATCGTCTCCAATAAATGCTCCTGGATGTCCTA
ATTCTGCTCGAATAAGAATTCTTAAAGAAGTTCCTACTATTCCTGCTCAA
GCTCCGAAAGATAAAATATAAAGTTTCCCAATATCTTTATGATTKGTTGG
ACCCATTA

4. *Culex quinquefasciatus*Mitochondrial Cytochrome oxidase c subunit 1 sequences

> Nucleotide sequences of Culex quinquefasciatus detected at Kanyama (702)

CTTTAAAAAAGGGTTCCTGTTGCGTTTCGATATCCTCCTCCTCAATGGAA
GGGGGAAGGGCCCTATATAATTTCGTCAGAGAAGAGATAGTAATAGCAC
CAGCTAAAACAGGTAAAGAAAGAAGCAATAAAATGGAAAAATAACTAC
TGATCAAACAAAGAAAGGTATTCGATCAAGAGTAATTCCTGAGGATCGT
ATATTAATTACTGTTGTAATAAAATTTACTGCACCTAAAATTGATGAAAT
TCCTGCTAAATGTAAAGAAAAAATAGCTAAGTCTACTGAAGCTCCAGCAT
GAGCTGTTCTAGATTAAAGAGGGGGATACACTGTTCGTCCATTCCCAGCC
CCATTTTCTACTAAACTACTTTAAAGTCGTAGTGTCAATGAAGGAGGTCG
TATTCAAAAACTTATATTAATTATTCGAGGAAAGACCATATCTGGAGCTC
CTAAATTAAAGGAAACTAATCAATTTCCAAGTCCGCCATTATGATTGGTA
TTACTATAAAAAAAARKAAAAAGAAGCATGGGCAGTTACATAAATTATT
AATTTATCATTTCCAATACTTACACCTGGGTTACTTAWGGTCTGCTCGRA

TTAGGAACTTTAAAGAAGTCCCACCTATTTCCAGSTCYAACCCMTCAAAA
ATAAATATAATTGTTCCATTATCTTTGATGAATTGYTGACCCAATGTGTGT
GTTTGG