BLOOD MEAL ANALYSIS AND ARBOVIRUS DETECTION IN A SYLVATIC *AEDES CHAUSSIERI* AT OLOOLUA FOREST, KENYA

BY

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THIS THESIS IS SUBMITTED IN PARTIAL FULFILMENT FOR THE AWARD OF DEGREE OF MASTERS OF SCIENCE IN MEDICAL AND VETERINARY ENTOMOLOGY AT THE UNIVERSITY OF NAIROBI.

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DECLARATION.

This is to testify that this research thesis is my original work and has never been presented for the award of degree in any university.

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DEDICATION

This thesis is dedicated to my entire family members. My lovely wife Aphlyne, my son Brayden and lovely daughters; Breanah and Brealyn. I dedicate this work also to my late parents Mr. and Mrs. Dancun Kisero and the entire Adoda family.

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ABBREVIATIONS AND ACRONYMS

BLAST – Basic Local Alignment Search Tool

CHIKV – Chikungunya Virus

CLB – Cell Lysis Buffer

CPE – Cytopathic Effect

DENV – Dengue Virus

DNA – Deoxyribonucleic Acid

DPBS - Dulbecco's Phosphate Buffered Saline

EDTA - Ethylenediaminetetraacetic Acid

EIPs – Extrinsic Incubation Periods

HRM – High Resolution Melt

i.e. – That is

ICA – International Catalogue of Arboviruses

icipe – International Centre of Insect Physiology and Ecology

MEM – Minimum Essential Medium

MLEID_LAB – Martin Luscher Emerging Infectious Diseases Laboratory

PPS – Protein Precipitation Buffer

rcf – Relative Centrifugal Force

RNA	**-** Ribonucleic Acid
rpm	– Revolutions Per Minute
RT- PCR	– Real Time Polymerase Chain Reaction
RVFV	– Rift Valley Fever Virus
VC	– Vector competence / Vectorial Capacity
WHO	**–** Word Health Organization
WNV	– West Nile Virus
YFV	– Yellow Fever Virus

ABSTRACT

Arboviral pathogens are a growing public health problem especially in Africa. Bridge vectors facilitate virus transmission to humans from forest or sylvatic environments although the species involved are not fully understood. In this study, we investigated the role of *Aedes chaussieri* in sylvatic arboviral transmission cycle. Archived samples collected from the Oloolua Forest in the outskirts of Nairobi, Kenya, were analyzed in pools for viruses by cell culture in Vero Cells followed by RT-PCR and sequencing. Blood-fed specimens were processed for host meal sources by PCR and sequencing of a portion of the mitochondrial 12S ribosomal RNA gene. Viral screening revealed West Nile virus (WNV) infection in two pools after cell culture and subsequent molecular identification using primers targeting polyprotein gene specific to Flaviviridae family. Sequences of the isolates (259-268 bp) were 99% identical to each other and closely related to a lineage 1 WNV strain isolated from the mosquito *Culex poicilipes* in Senegal (98% identity). Blood meal patterns revealed overwhelming feeding on tree hyrax (70.9%, 197/328), followed by antelope (16.2%, 45/328), human (8.6%, 24/328) and minor representation of other avian and mammalian wildlife species. The results unravel novel association of WNV with *Ae. chaussieri* that not only feeds largely on wildlife species but also humans, thus, can serve as potential bridge vector of the virus. Further studies are recommended to understand the vectorial capacity of this mosquito species.

1.0 INTODUCTION

Arthropod-borne viruses otherwise called arboviruses, are characterized by a biological cycle in which they replicate in blood-feeding arthropods notably mosquitoes, sand flies, ticks and biting midges (Sang, *et al.*, 2001). All arboviruses circulate among wildlife, and many cause disease to humans and livestock that are incidental or dead-end hosts, after spillover transmission from zoonotic cycles (Weaver, *et al.*, 2010). Infections with arboviruses cause a range of clinical syndromes in humans, depending on the infecting virus, ranging from a self-limiting, febrile illness to life-threatening encephalitis or hemorrhagic fever.

A significant proportion of the world's population is at risk of arbovirus infection (Wilder-Smith, *et al.*, 2017), reminiscent of the expanding range of vector-borne diseases (Torto, *et al.*, 2021). In the last two decades, there has been an upsurge in arbovirus activities globally, with reported outbreaks in various parts of the globe. Examples include Zika in the Philippines, Brazil and USA in 2012, 2015 and 2016 (Chang, *et al.*, 2016); Chikungunya in Italy in 2007 and 2017 (Caputo, *et al.*, 2020), Reunion Island between 2005-2006 (Renault, *et al.*, 2007). Others include Chikungunya (Konongoi, *et al.*, 2018) and dengue outbreaks including confirmed laboratory cases in Kenya (Lutomiah, *et al.*, 2016; Muthanje, *et al.*, 2022; Obonyo, *et al.*, 2018) and Yellow fever in Brazil and the WHO African Region (Figueiredo, *et al.*, 2020). The frequent arbovirus activities have been facilitated by a myriad of factors amongst them climate change, increased international travels, rapid urbanization and high human population growth leading to human encroachment into mosquito dwelling forested areas (Esser, *et al.*, 2019).

1.1 Problem statement

Mosquito-borne surveillance remains a critical component of arbovirus disease risk assessment. This entails analyzing mosquitoes for early warning of virus presence, mapping out of high-risk areas that could be targeted for control to reduce potential for human disease. Mosquitoes like other arthropods once infected following a viremic blood meal on vertebrate hosts, remain infected for life. In Kenya, active surveillance has demonstrated virus activities in diverse mosquito species belonging to the genera *Aedes*, *Anopheles*, *Culex* and *Mansonia* (Chiuya, *et al.*, 2021; Ochieng, *et al.*, 2013; Sang, *et al.*, 2017a). Few surveillance studies have been conducted in sylvatic environments, despite being the origin for most arboviruses where they circulate among wildlife and mosquitoes. Arbovirus emergence in humans could result from spillover transmission from the sylvatic cycle via bridge vectors. Knowledge of sylvatic vector species and distribution and associated pathogens can improve understanding of ecological foci and enzootic cycles of pathogens of potential medical and veterinary health importance (Eastwood, *et al.*, 2020).

1.2 Justification

Aedes chaussieri first described by Edwards (Edwards, 1923) is a sylvatic mosquito species. Not much is known about its biology although few studies in Kenya have reported its occurrence in different ecological areas (Eastwood, *et al.*, 2020; Tchouassi, *et al.*, 2019a). A recent study found this species to be predominant in trap catches of adult mosquito fauna including engorged cohorts, in the Oloolua Forest located in the outskirts of Nairobi, the Kenyan capital city (Tchouassi, *et al.*, 2019a). The forest houses the Institute of Primate Research that conducts biomedical research primarily on non-human primates. Thus, mosquito-human biting activity could pose risk of transmission of arboviruses. In this study, towards determining the arboviral disease risk, we

assessed the role of *Ae. chaussieri* in sylvatic arbovirus transmission given its significant abundance and blood feeding activity. Specifically, we screened the mosquito for arbovirus presence and determined the blood feeding patterns. Thus, this work sought to close the knowledge gap in sylvatic mosquito species diversity, arboviral transmission cycles and opens the window for further investigation on other less known sylvatic mosquito species other than *Aedes chaussieri* and their potential role(s) in the transmission and maintenance of arboviruses.

1.3 Null Hypotheses

1. *Aedes chaussieri* is an opportunistic feeder.

2. *Aedes chaussieri* plays a role in the maintenance and/or circulation of arboviruses in sylvatic environments in Kenya.

1.4 Objectives

1.4.1 General objective

To determine the role of *Aedes chaussieri* in sylvatic circulation and maintenance of arboviruses in Oloolua Forest, Kenya.

1.42 Specific objectives

1. To determine the *Aedes chaussieri* blood meal vertebrate host source.
2. To screen *Aedes chaussieri* for viruses

2.0 LITERATURE REVIEW

2.1. Overview of medically important arboviruses

Arboviruses are a group of biological viruses transmitted by blood feeding arthropods such as mosquitoes, ticks, sandflies and biting midges (Gubler, 2002). However, these viruses are maintained in nature via different mechanisms such as; (i) through infections developed by the vector in the process of feeding from viremic hosts, (ii) transovarial and (iii) transstadial passage of virus from parent to offsprings in vectors. For majority of these viruses, humans become dead end hosts when accidentally infected with very limited information available on direct human to human transmission cases. However, there are deviations whereby the virus could be transmitted from one infected human to the other via vectors as in the case of Dengue and Zika viruses.

In the the International Catalogue of Arboviruses (ICA), there are more than five hundred reported arboviruses. However, this number is ever growing and renaming of several families is currently being undertaken (Kuhn, *et al.*, 2021; Marchi, *et al.*, 2018). Arboviruses affecting both humans and livestock are found in three broad families of *Flaviviridae, Togaviridae* and *Bunyaviridae* (Young, 2018).

2.1.1 Flaviviridae

Flaviviruses consist of 53 recognized viruses out of which 40 are known to cause human diseases (Best, 2016). Some of the most notable diseases caused by flaviviruses include Yellow fever, West Nile, dengue fever, Japanese encephalitis and Zika viruses (Guggemos, *et al.*, 2021). Globally, there have been increased outbreaks of arbovirus diseases in the recent past among them those caused by flaviviruses. Examples include but not limited to: Zika outbreaks in French Polynesia

in 2013 and Brazil in 2015 (WHO, 2018), Germany in 2013-2014 and Australia in 2015 (Chang, *et al.*, 2016),Yellow fever outbreaks and confirmed laboratory cases have been reported in the recent past from different global regions such as Cameroon, Central African Republic, Chad, Côte d'Ivoire, Democratic Republic of Congo, Gabon, Ghana, Nigeria, and Republic of the Congo - reported a total of 151 confirmed cases of yellow fever. In 2022, Yellow fever outbreak and confirmed cases were reported in Kenya and Uganda (WHO, 2022). West Nile virus however is the most widespread of all the flaviviruses globally. This could be as result of the fact that its' reservoir host (birds) do have frequent mobility from place to place through migratory nature they exhibit (Ain-najwa, *et al.*, 2020). The economic burden of flaviviruses in the last decade globally has been enormous. For instance, for dengue alone, it is reported that there occurs approximately 390 million infections annually (as of 2019) with at least 96 million infections proceeding to clinical manifestation (WHO, 2021a; Yang, *et al.*, 2021).Thus in terms of severity and distribution, flaviviruses are considered the most severe and widely distributed affecting both humans and livestock hence posing great global public health concern.

2.1.2 Togoviridae

This family comprises two major genera *viz*; genus *Alphavirus* and genus *Rubivirus*. Viruses in the genus *Rubivirus*, such as Rubella (German measles) are not considered arboviruses since they infect only humans and primarily transmitted via direct droplets and respiratory route (Parkman, 1996). The genus *Alphavirus* encompasses more than 30 virus species of which chikungunya virus is the most important medically. Most of the viruses in the genus *Alphavirus* cannot develop sufficient viremia in humans to infect mosquitoes (Chen, *et al.*, 2018). The most notable virus affecting humans from this family is Chikungunya virus. Chikungunya virus was first isolated in the Makonde Plateau in Tanzania in 1952/53 (Battisti, *et al.*, 2021). Globally, chikungunya virus

has been reported in several countries from the continents of Africa, Europe, Asia, the Americas and Oceania/Pacific Islands (CDC, 2022; Clements, *et al.*, 2019). Recent outbreaks and confirmed cases include but not limited to: Italy and France (2017), Sudan (2018), Yemen (2019), Cambodia and Chad (2020) (WHO, 2020). In Kenya and east Africa, the most recent chikungunya outbreak was reported in Mombasa in 2017/2018 (Eyase, *et al.*, 2020). Previous seroprevalence studies have confirmed the presence of IgG antibodies against chikungunya virus in humans in coastal Kenya (LaBeaud, *et al.*, 2015). Furthermore, chikungunya virus has been isolated from field collected mosquitoes in both urban and rural areas in Kenya; western Kenya and Coastal regions (Heath, *et al.*, 2020) indicating active circulation of the virus during inter-epidemic periods. In Uganda, an antibody specific to chikungunya virus prevalence study carried out from five regions distributed throughout Uganda, 31.7% of all the samples tested were positive of CHIKV IgG antibody which was the highest (Clements, *et al.*, 2019). This studied showed that there is circulation of the virus which normally go undetected since the blood samples were drawn from healthy people. In Tanzania, a similar study as that in Uganda still showed circulation of chikungunya within the population (Faustine, *et al.*, 2017).

2.1.3 Bunyaviridae

This family is divided into five major genera: *Orthobunyavirus* (largest with about 150 viruses), *Phlebovirus*, *Nairovirus*, *Hantavirus (*transmitted via rodents*)* and *Tospovirus* (CDC, 2012). They are transmitted to humans by bites of infected mosquitoes, ticks and midges and have animals like rodents and livestock as their reservoirs hosts (Odhiambo, *et al.*, 2016), with the exception of Hantavirus which is rodent-borne. Globally, virus belonging to this family have been reported in various parts of the world such as Rift Valley Fever virus in: Yemen and Saudi Arabia (2000), Kenya, Tanzania and Somalia (2006), South Africa and Mauritania (2010), South Africa and

Namibia (2009-2011), Mauritania (2012 and 2015), Mauritania and Senegal (2013-2015), Angola and Niger (2016), Gambia (2017), Kenya (2018), France (2019) and Uganda (2016 to 2020) (Métras, *et al.*, 2020). Other RVF outbreaks include: Tanzania in 2007 (Sindato, *et al.*, 2011) and in Arabian Peninsula (Kuwait, Oman, Qatar, Saudi Arabia and United Arab Emirates countries) in 2000 (Rolin, *et al.*, 2013). Ngari virus which is also a member of Bunyavirdae family has been reported either as an outbreak or confirmed detection from mosquito pools in various parts of the world. Most notably: the outbreak of febrile illness in Sudan, Kenya, Tanzania and Somalia as well as from goat and sheep in Mauritania (Braack, *et al.*, 2018) and the isolated Ngari virus from pools of mosquitoes in Senegal in 2013 (Ndiaye, *et al.*, 2018).

2.2 Arbovirus transmission cycles.

There are basically three modes of arbovirus transmission cycles involving both maintenance and amplification. These are: horizontal, vertical and venereal transmission cycles. Briefly, horizontal transmission the infected mosquito transmits the virus to a host during blood meal, the host becomes viremic and when fed upon by another mosquito, the virus is picked and transmitted to the next host hence the cycle continues (Agarwal, *et al.*, 2017). Vertical transmission involves the passage of virus from one generation of mosquito to the next one via the eggs. The infected eggs eventually pass through the stages of development to adult mosquito which then becomes infected with the virus naturally (Emanuel, *et al.*, 2012). Venereal transmission involves the transfer of virus from male to female mosquitoes through copulation. Though it's not easy to pin point whether the female had acquired the virus orally or venereally, in literature, some experiments have suggested this to occur in alphaviruses, flaviviruses and bunyaviruses as had been reported in the case of chikungunya (Mavale, *et al.*, 2010).

Sylvatic transmission cycle, which basically involves the infection of naïve non-human primates by infected arboreal mosquito species from viremic infected non-human primates has been documented (Valentine, *et al.*, 2019a). Typically, these non-human primates reservoir of arbovirus infection and show no clinical signs of infection hence help maintain the virus in nature (Jones, *et al.*, 2008). Two scenarios exist where people might contract these viruses: (i) when they invade forested areas and come into contact with infected forest dwelling mosquitoes and (ii) when infected arboreal mosquitoes move into settlement regions in search of blood meal (Valentine, *et al.*, 2019b). Local transmission of the virus then occurs when viremic humans (infected previously by forest dwelling mosquitoes) move into urban areas and are fed upon by urban dwelling mosquitoes which in turn spread the virus to uninfected population within the urban area thus it is said that sylvatic transmission has "spilled over" into "urban transmission cycle" (Hanley, *et al.*, 2013; Vasilakis, *et al.*, 2011; Weaver, 2005).

2.3 Arbovirus vector profile

The vector profile for several arbovirus diseases vary greatly. Some appear to have narrow host range while others don't. Several studies have reported different vectors for different arbovirus diseases. For example, in the case of dengue fever, there could be unidentified potential vectors though the widely reported vectors are *Aedes aegypti* and *Aedes albopictus* with the latter serving as the secondary vector (Higa, 2011; WHO, 2021b). During the 2014 dengue fever outbreak in Tokyo, Japan, 16 pools of *Aedes albopictus* collected tested positive for dengue virus (Moi, *et al.*, 2016). The same vectors of dengue (*Aedes aegypti* and *Aedes albopictus*) have been incriminated as the vectors for chikungunya as well. During the 2005-2006 chikungunya outbreak in the La Reunion in the Indian Ocean, Aedes albopictus species was identified as the main vector (Robinson, *et al.*, 2014; Vairo, *et al.*, 2019; Yakob, *et al.*, 2013). Sylvatic chikungunya is

transmitted primarily by the sylvatic arboreal mosquito species such as *Aedes furcifer* and *Aedes africanus* and majorly rely on the non-human primates as the reservoir hosts (Lo Presti, *et al.*, 2014). Zika virus on the other hand has been reported to be transmitted by Aedes aegypti in the laboratory experiments (Bolling, *et al.*, 2015; Huang, *et al.*, 2017), in the field (Cevallos, *et al.*, 2018) and Aedes albopictus in the laboratory experiment (Chouin-Carneiro, *et al.*, 2016; Ciota, *et al.*, 2017; Jones, *et al.*, 2020). Several mosquito species from the genus *Culex* have been implicated as the principal vectors of West Nile virus such as Culex vittatus (LaBeaud, *et al.*, 2011; Ochieng, *et al.*, 2013) with *Culex pipiens* being the most widely considered primary vector (Fortuna, *et al.*, 2015). However, there has been detection 0f West Nile virus in other mosquito species other than *Culex* such as *Aedes mcintoshi* in Kenya (Sang, *et al.*, 2017b) which conforms to this work where the same was detected in a pool of *Aedes chaussieri*.

2.4 Concept of arbovirus surveillance and strategies

Surveillance is critical in arbovirus disease prevention and control as it provides an indication of periods of elevated or emergent virus activities hence serves as a warning system that may be used to take necessary measures to limit the intensity and length of epidemics (Ramírez, *et al.*, 2018). Three main strategies are commonly employed in the surveillance of arboviruses. They include monitoring human and animal diseases, vertebrate host arbovirus surveillance and mosquito-based arbovirus surveillance (Ramírez, *et al.*, 2018).

2.4.1 Human and animal disease monitoring

This surveillance approach is passive and relies heavily on hospital data, confirmed laboratory cases as well as information from various health practitioners to the general public concerning the emergence or re-emergence of a particular arboviral disease. Various systems are used in different nations to monitor arboviruses, such as the national arbovirus surveillance system (ArboNET) in

the USA (Hadler, *et al.*, 2015) and National Notifiable Disease Surveillance System in Australia (NDDS, 2015). A major limitation of this surveillance method is that in less developed countries, laboratory confirmed testing is not readily available. Also, most diagnosis of arbovirus rely on clinical symptoms that may overlap between arboviruses and other pathogens, which are non-arboviruses

2.4.2 Use of sentinel animals (Vertebrate host arbovirus surveillance)

This involves employing sentinel animals as indicators of virus activity that could be an elevated risk to both humans and targeted animals (Halliday, *et al.*, 2007). The immunologically naïve animals are taken to a specific location, bled periodically and tested for the presence of virus specific antibody. When positive, this would mean that the animal was exposed to the virus indicative of circulation within the area. Different species of sentinel animals are used for different viruses, for instance, chicken is used to monitor circulation of West Nile virus as commonly deployed in the USA (Reisen, *et al.*, 2000). Even though this method shows timely detection of arbovirus circulation, it is coupled with some challenges as well. First, it is not easy to determine the specific foci from which the local enzootic arbovirus is found. Secondly, other animals serve as amplifying hosts for some arboviruses as is the case of pigs for Japanese encephalitis virus, thus, the risk of transmission to humans (Van Den Hurk, *et al.*, 2008). Additionally, the cost of rearing these sentinel animals could be high thus hindering the effective deployment for monitoring arbovirus activities (Hall, *et al.*, 2012). Finally, very closely related viruses such as Japanese encephalitis virus, West Nile virus and Murray Valley encephalitis virus can cross-react hence requiring additional methods to unequivocally point at the results (Scott, *et al.*, 2001).

2.4.3 Mosquitoes (Vector) based arbovirus surveillance

Mosquito-based arbovirus surveillance is considered more reliable and accurate method of arbovirus surveillance compared to the previous two; that is, human and animal disease monitoring and the use of sentinel animals. Even though it also has some few drawbacks, its benefits outweigh the drawbacks compared to the two above since it indicates the presence of active circulation of the virus under study (Ndiaye, *et al.*, 2018). Mosquito-based surveillance can detect seasonal commencement or increase in viral circulation, thus, can be utilized as an early warning system for accurate implementation to curb the potential outbreak (Ndiaye, *et al.*, 2018). Mosquito-based virus surveillance techniques are especially useful in the study of eco-epidemiology and viral transmission since they allow the identification of possible vectors of a certain virus as well as the virus's spatiotemporal dynamics. Mosquito monitoring can also aid in the discovery of novel arbovirus species of medical and veterinary significance (Gu, *et al.*, 2008; Van Den Hurk, *et al.*, 2012; Ochieng, *et al.*, 2007). This approach of mosquito surveillance conveys information on active circulation of arbovirus in the environnement unlike the previous two, thus, considered more accurate technique/tool in arbovirus disease surveillance.

2.5 Vectorial capacity/competence and blood meal host preference

Vectorial capacity (VC) is a basic entomological restatement of a pathogen's specific reproductive rate, defined as the amount of secondary infections predicted to result from the introduction of a single infection in a naive population (Lounibos, *et al.*, 2016). Some of the factors that determine the vector capacity of mosquitoes include: survival of the mosquito, mosquito density, the feeding rate of infected mosquitoes, length of extrinsic incubation period and vector susceptibility to a given virus(Chepkorir, *et al.*, 2014). Vector competence directly determines how effectively a

mosquito picks up a virus, harbors it and support its development and subsequently transmit it to a susceptible host (Mack, 2001).

Blood feeding is critical for mosquitoes in two ways: for providing nutrients needed for egg production in adult mosquitoes and development and progression of immature life stages (Brackney, *et al.*, 2021). Blood feeding directly determines the success of mosquito pathogen transfer from the vector to host(s). During blood meal, mosquitos ejects out the virus together with saliva into the host. This therefore provides an ideal route for virus entry into the host's circulation system. It is thus critical to study the different patterns of blood feeding and blood feeding behaviors of mosquito vectors for it provides insight into the possible host range for various arbovirus diseases and potential outbreaks and/or infections. Moreover, blood feeding pattern of a given mosquito species can determine the spatial distribution of the disease(s) transmitted by that particular species (Richards, *et al.*, 2006).

Basically, there are different categories of blood feeding habits exhibited by different mosquitoes. These are zoophilic, anthropophilic and generalists. Briefly, zoophilic majorly prefers feeding on animals most of the malaria vectors tropical countries have shown this characteristic (Kiware, *et al.*, 2012). They, however, thrive in areas that have experienced heavy insecticide spraying season resulting into suppressed anthropophilic species (Bayoh, *et al.*, 2010). Anthropophilic feeding involves preference of mosquito entirely to humans. For instance, species such as *Anopheles gambiae* shows very consistent preference to human blood (Stone, *et al.*, 2018). Lastly, many mosquito species such as *Culex quinquefasciatus* show generalist feeding habit with opportunistic aspect when there occurs a variation and /or availability of host(s) (Takken, *et al.*, 2013). However, there is always an element of opportunistic feeding in each category.

2.5.1 Factors affecting blood feeding patterns in mosquitoes.

The ability of a mosquito to choose a particular host for its' blood meal depends on several factors. These include: season, habitat where the sample collection was carried out, host abundance and diversity. Season of the year affects the distribution and abundance of a particular host from which the vector feeds from. For instance, a study carried out by Kent et al., to determine blood meal source for *Culex tarsalis* in Colorado in 2007 showed a varied hosts preference from the month of June through to September (Kent, *et al.,* 2009). The distinctive feeding relationships that each mosquito species has with various vertebrates point to species-specific feeding habits. This hence, dictates the choice of blood for a given mosquito species within the same habitat (Stephenson, *et al.*, 2019).

2.5.2 Mosquito blood meal analysis methods

There are several methods that have been employed in analyzing blood meal sources from which the mosquito might have fed. Basically, there are two major techniques that have been deployed to determine blood meal sources of hematophagous arthropods. These are: serologic techniques such as precipitin test, latex agglutination and ELISA (enzyme-linked immunosorbent assay), molecular technique (majorly polymerase chain reaction) (Santos, *et al.*, 2019) and mass spectrometry (Hlavackova, *et al.*, 2019). Although not commonly practiced recently, serologic techniques were very much important in the early days in trying to understand the dynamics between the host, pathogen and vectors in determining the potential of disease transmission (Serology, 1975). The mostly widely used techniques currently in determining the various blood meal sources of various blood feeding arthropods are molecular (polymerase chain reaction based) techniques as had been applied in determining blood meal source for tsetse fly *(Glossina palidipes*) (Channumsin, *et al.*, 2021), mosquito blood meal identification studies (Keven, *et al.*, 2020; Musa, *et al.*, 2020; Muturi, *et al.*, 2021; Santos, *et al.*, 2019), ticks blood meal analysis studies (Allan, *et*

al., 2010; Lah, *et al.*, 2015; Wang'ang'a Oundo, *et al.*, 2020) and sources of blood meal for sandflies (Jaouadi, *et al.*, 2018; Owino, *et al.*, 2021; Sales, *et al.*, 2015).

2.6 Drivers of arbovirus risks

Both biotic and abiotic factors influence the transmission and spread of arboviruses. These factors include: increased human and animal populations, as well as habitat changes and globalization (Esser, *et al.*, 2019).

2.6.1 Biotic factors

Human driven factors such as increased human population growth, urbanization and trade have a direct impact on mosquitoes. Increased human population growth exerts pressure on the natural resources such as land. This in turn leads to encroachment to forests, which are natural habitats for mosquitoes for human settlement (Cropper, *et al.*, 1994). This thus leads to more exposure of humans to forest dwelling mosquitoes that may be potential vectors of various arboviruses. Urbanization, which yields urban centres characterized by old tyres and abandoned containers that acts as breeding sites for most mosquitoes such as *Aedes.* As a result, this would lead to increased rate of mosquito-human interaction hence elevated risk of arboviral disease infection and transmission (Wilke, *et al.*, 2019). Trade, which involves movement of people and objects from one place to the next, has been incriminated as a source of emergence for various arbovirus diseases in new areas other than their endemic regions. For instance, the introduction of Yellow fever in the Americas has been attributed to the movement of sailing ships from Africa along with *Aedes* mosquitoes during the slave trade era (Bryant, *et al.*, 2007).The ease of movement of people could also aid the local transmission of some arboviral diseases when viremic individuals are fed upon by competent mosquito vectors that in turn spread the virus to other individuals (Valentine, *et al.*, 2019a).

2.6.2 Abiotic factors

These environmentally driven factors determine the distribution, population and survival of mosquito vectors. Of notable influence are temperature and rainfall. Water availability dictates the ovipositional nature of mosquitoes. For instance, *Aedes aegypti* female prefers laying eggs in natural pools such as rock pools and tree holes as well as human generated water pools like abandoned containers and old tires (Powell, *et al.*, 2013). Presence of enough water in such places would therefore, accelerate the breeding of *Aedes* with an eventual increase in number thus an elevated rate of virus infection and transmission. Temperature is a critical component for viral infection and transmission (Ciota, *et al.*, 2019). Previous studies have suggested that temperature has a direct impact on various components of vectorial capacity. For instance, adult holding temperature has a considerable impact on vector competence for zoonotic arboviruses of concern. In ectothermic hosts, increases in ambient temperature boost virus replication rates. Because viral load is directly connected with viral dispersion in ectothermic hosts, temperature rises result in shorter extrinsic incubation periods (EIPs) and greater overall transmissibility (Ciota, *et al.*, 2019).

3.0 MATERIALS AND METHODS

3.1 Study site

Archived adult *Ae. chaussieri* mosquito samples (blood-fed and unfed) stored at -80^{o}C were used in this study. They were collected using CO_2-baited BG sentinel traps additionally augmented with host odors, in the Oloolua forest, located in the outskirts of Nairobi (Fig 1). The forest is located between the Kenyan counties of Nairobi and Kajiado. It has a wide variety of both mosquitoes and wildlife species and harbors the Institute of Primate Research (IPR). Details of the trapping design are described in Tchouassi *et al.,* (2019). However, the details of trap treatment, spacing, trapping method, time of collection and point coordinates are found in the previous work done in Oloolua (Tchouassi, *et al.,* 2019b).

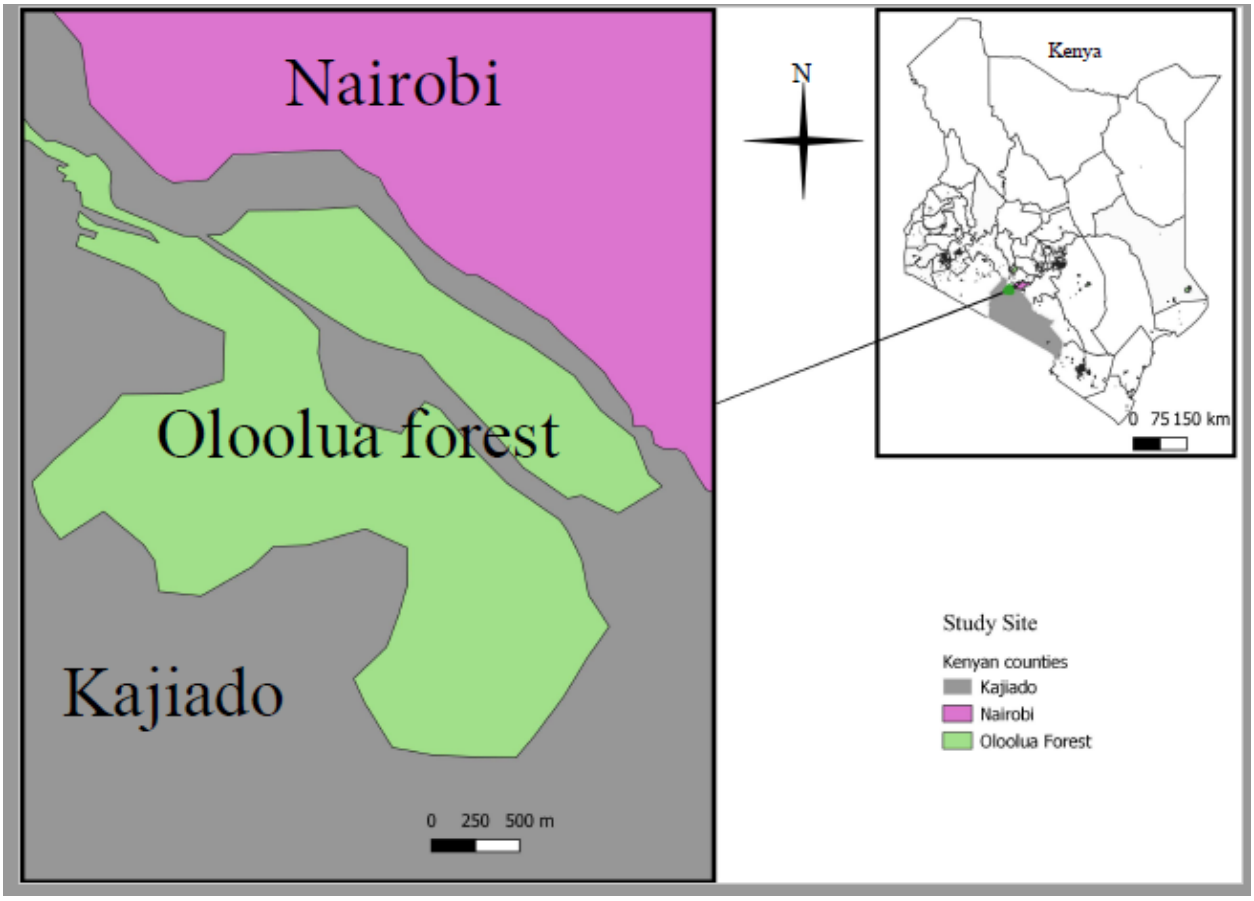

Figure 1. Study site (Oloolua Forest)

3.2 Identification of blood meal source.

3.2.1 Genomic DNA extraction

Blood fed mosquitoes were retrieved from -80^{0}C freezer and dissected using a sterile dissection pin and sharp pointed forceps. Individually engorged abdomen was separated from the rest of the body, placed in a sterile 1.5mL micro centrifuge tubes. Genomic DNA was extracted using laboratory optimized protein precipitation method as outlined previously (Wang'ang'a Oundo, *et al.*, 2020). Briefly, 300µL of Cell Lysis Buffer (10mM Tris-HCL [pH 8.0], 5mM EDTA, 0.5&SDS) added to the abdomen and squashed using Mini-bead beater (BioSpec Bartlesville, OK) for 30s and then incubated at 65^{o}C for 30min. Thenceforth, 100µL of Protein Precipitation Buffer

(8Mammonium acetate, 1mM EDTA) was added, vortexed for 30s, placed on ice for 30min before centrifuging at 25,000 relative centrifugal force (rcf) for 5min at 4°C. The pellets were discarded and 450µL of the supernatant transferred to a new 1.5mL sterile microcentrifuge tube with 300µL of absolute isopropanol, mixed thoroughly by inverting 100 times, then centrifuged at 25,000 relative centrifugal force (rcf) for 60min. The supernatant was discarded and 300µL of ice cold 70% ethanol added to each tube, mixed thoroughly by inverting 50 times and centrifuged at 25,000 relative centrifugal force (rcf) for 30min. The ethanol was pipetted out gently and the tubes inverted on a clean paper towel and left to air dry overnight. The DNA pellets were re-suspended using 60µL nuclease free water, incubated at 65^0C for 30min and stored at -20^0C awaiting polymerase chain reaction (PCR).

3.2.2 Genomic DNA amplification

Polymerase chain reaction (PCR) was carried out using primers targeting the mitochondrially encoded 12S ribosomal RNA vertebrate gene (Table 1). Briefly, this was done in a 20µL PCR reaction volume containing 1X Mytaq reaction buffer, 2 units of Mytaq DNA Polymerase enzyme (Meridian bioscience), 0.5µM concentration of both forward and reverse primers, 11.6µL of nuclease free water and 2µL of genomic DNA template. The thermal cycling conditions used were as follows: initial denaturation was done at 95^0C for 3min, followed by 40 cycles of denaturation at 95^0C for 20s, annealing at 59^0C for 30s and extension at 72^0C for 30s followed by a final extension at 72^0C for 7min. The PCR was carried out using a 96 well, 0.2mL, 3-zoned VeriFlex Block SimplAmp Thermal Cycler (Thermo Fisher Scientific, MA).

3.2.3 Gel electrophoresis

The PCR products were then run in agarose gel electrophoresis alongside 100bp DNA ladder (Solis Biodyne) for 40min at 300mA/120V in 1.5% ethidium stained agarose gel in Tris-borate ethylenediaminetetraacetic acid (EDTA) buffer (see appendix 1). Bands of interests were noted, and their respective amplicons cleaned using ExoSAP-IT kit (USB Corporation, Cleveland, OH) following manufacturer protocol and outsourced for sequencing at Macrogen Europe BV, Amsterdam, Netherlands.

3.3 Identification of arboviruses

3.3.1 Homogenization

Pools of 25 mosquitoes (head and thorax) for the fed and pools of 1-25 for the unfed mosquitoes were homogenized for 30s in a sterile micro centrifuge containing 750mg of 2-mm tria-stabalised zirconium oxide beads using 500-1000µL 1X Dulbecco's Phosphate Buffered Saline (DPBS) (giblico) on mini-bead-beater (BioSpec Bartlesville, OK) and on bench top centrifuge (Eppendorf Centrifuge 5417R) at 2,000 revolutions per minute for 15min at 4^0C. From the supernatant, 500µL was transferred to a clean sterile 1.5mL microcentrifuge tube from which 20µL was then picked from 10 pools to constitute 200µL of super-pool.

3.3.2 Cell Culture

Vero cells were grown in minimum essential medium (MEM) with Earle's salts and reduced Sodium bicarbonate ($NaHCO_3$), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 2% L-glutamine and 2% antibiotic/antimycotic solution with 10,000 units' penicillin, 10mg streptomycin and 25µg amphotericin B per mL and incubated at 37^0C in 5% CO_2 overnight. Each super-pool homogenate was centrifuged at 12,000 rpm (Eppendorf centrifuge 5417R) for 10

minutes at 4^0C. The supernatant (50µL) was inoculated in Vero cells in 22 of the 24 well plate grown in MEM, supplemented with 2% FBS, 2% L-glutamine and 2% antibiotic/antimycotic, with the remaining 2 wells acting as negative control. The negative control wells were filled with only media containing the Vero cells. The plates were incubated at 37^0C at 5% CO_2 incubator with frequent rocking after every 15min for virus adsorption. A volume of 1mL MEM supplemented with 2% FBS, 2% L-glutamine and 2% antibiotic/antimycotic) was then added and incubated at 37^0C at 5% CO_2. This was thereafter observed daily for a period of 14 days for any cytopathic effect (CPE) (Mulwa, *et al.*, 2018). All the samples with CPE were harvested and RNA extracted as described below.

3.3.3 RNA extraction

3.3.3.1 RNA extraction from fed samples

RNA was extracted from single fed samples using Qiagen QIAamp®Viral RNA Mini Kit (QIAGEN GmbH, QIAGEN Strasse 1, 40724, Hilden, Germany) following manufacture's protocol. Briefly, in a clean sterile 1.5mL micro-centrifuge tube containing the abdomen of the fed mosquito sample, 560µL of freshly prepared buffer AVL containing carrier RNA was added and ground using pestles. This was then incubated at room temperature (25^0C) for 10mins before centrifuging briefly to remove drops from inside the lid. In addition, 560µL molecular grade ethanol (96-100%) was added to the mixture and pulse vortexed for15s after which centrifuged briefly. In a clean sterile spin column inserted in a 2mL collection, 630µL of the solution above was applied with a lot of caution not to wet the rim and centrifuged at 8000rpm for 1min. This step was repeated until all the samples have gone through the spin column. The first wash was done using 500µL of buffer AW1 centrifuged at 8000rpm for 1min and the final wash using 500µL buffer AW2 centrifuged at 14,000rpm for 3mins. A dry spin was done at top speed for 3mins to

remove any residues of ethanol. Double elution was done using 30µL each of buffer AVE and centrifuged at 8000rpm for 1min.

3.3.3.2 RNA extraction from super-pools (Cell culture)

RNA was extracted from the 200µL super-pool using Qiagen QIAamp®Viral RNA Mini Kit (QIAGEN GmbH, QIAGEN Strasse 1,40724, Hilden, Germany) following the manufacturer's protocol with minor modifications. Briefly; 800µL of freshly prepared buffer AVL containing carrier RNA was pipetted into a sterilized 1.5ml microcentrifuge tube. 200µL cell homogenate was added to the microcentrifuge tube containing buffer AVL- carrier RNA and mixed thoroughly by pulse vortexing for 15s. This was then incubated at 25^0C for 10 minutes and centrifuged briefly for 30 seconds at 8000rpm to remove drops from the inside of the lid before adding 560µL of molecular grade absolute ethanol (96-100%) and mixed thoroughly by pulse vortexing for 15s and centrifuging at 8000rpm to remove the drops from inside the lid. From this solution, 630µL was carefully applied to QIAamp Mini column in a clean 2ml collection tube, centrifuged at 8000rpm for 1min and the tube containing the filtrate discarded. This was repeated until all the sample volume has been loaded into the spin column. Washing was done by using buffer AW1 where 500µL was carefully added to the spin column and centrifuged at 8000rpm for 1min, filtrate discarded and spin column placed in a new 2ml collection tube. The second wash was done using 500µL buffer AW2, centrifuged at 14000rpm for 3min. Dry spin was done by centrifuging the spin column placed in a new 2ml collection tube for 2 min at top speed to remove any residuals of ethanol. Double elution was done using buffer AVE where 30µL was loaded into a spin column, centrifuged for 1min at 8000rpm. This step was repeated to yield high quality RNA.

3.3.4 cDNA synthesis

The RNA extracts were immediately reverse transcribed to cDNA using SensiFAST ™ cDNA Synthesis Kit using 10µL of the RNA in a 20µL reaction following the manufacture's protocol. Briefly, in a sterile PCR strip tube containing 4µL 5X TransAmp Buffer, 1µL Reverse Transcriptase enzyme and 5µL RNase free water, 10µL of extracted RNA was added. The synthesis conditions were as follows: primer annealing at 25^0C for 10 minutes followed by reverse transcription at 42^0C for 15 minutes and lastly inactivation at 85^0C for 5 minutes.

3.3.5 Viral RNA amplification

3.3.5.1 Flavivirus screening

Flaviviruses were screened using Fu1F and CFD2R primers targeting the polyprotein gene specific to Flaviviridae family (Table 1) in a 20µL reaction volume containing 1X Mytaq reaction buffer, 2 units of Mytaq DNA Polymerase enzyme (Meridian bioscience), 0.5µM concentration of both forward and reverse primers, 11.6µL of nuclease free water and 2µL of cDNA template. The thermal cycling conditions used were as follows: initial denaturation was done at 95^0C for 10 minutes, followed by 35 cycles of denaturation at 95^0C for 30 seconds, annealing at 55^0C for 30 seconds and extension at 68^0C for 45 seconds followed by a final extension at 72^0C for 7 minutes.

3.3.5.2 Alphaviruses screening

For alphaviruses, the screening was done using VIR2052F and VIR2052R primers (Table 1) in a 20µL reaction volume containing 1X Mytaq reaction buffer, 2 units of Mytaq DNA Polymerase enzyme (Meridian bioscience), 0.5µM concentration of both forward and reverse primers, 11.6µL of nuclease free water and 2µL of cDNA template. The thermal cycling conditions used were as follows: initial denaturation was done at 95^0C for 10 minutes, followed by 35 cycles of

denaturation at 95^0C for 30 seconds, annealing at 49^0C for 30 seconds and extension at 72^0C for 30 seconds followed by a final extension at 72^0C for 7 minutes.

3.3.5.3 Phlebovirus screening

Phleboviruses were tested by generic nested PCR using Pan Phlebo primers Ph_F1, Ph_R1, Ph_F2 and Ph_R2 (Table 1) targeting conserved region of RdRp gene of Phleboviruses in 20µL reaction volume containing 1X Mytaq reaction buffer, 2 units of Mytaq DNA Polymerase enzyme (Meridian bioscience), 0.5µM concentration of both forward and reverse primers, 11.6µL of nuclease free water and 2µL of cDNA template for the first reaction before nesting and following thermocycling conditions as published previously (Marklewitz, *et al.*, 2019). Briefly, the thermal cycling conditions were: 10 cycles of 95^0C for 3 min, 94^0C for 15 s, 55^0C for 20 s and 72^0C for 30 s, then 30 cycles from 95^0C to 15 s, 50^0C to 20 s, 72^0C to 30 s and a final extension step of 72^0C for 5 min.

3.3.6 Gel electrophoresis

The PCR products were then visualized in agarose gel electrophoresis alongside 100bp DNA ladder (Solis Biodyne) for 40 minutes at 300mA/120V in 1.5% ethidium stained agarose gel in Tris-borate ethylenediaminetetraacetic acid (EDTA) buffer (see appendix 2). Bands of interests were noted, and their respective amplicons cleaned using ExoSAP-IT kit (USB Corporation, Cleveland, OH) following manufacturer protocol and outsourced for sequencing at Macrogen Europe BV, Amsterdam, Netherlands.

3.3.7 Sequence cleaning and analysis

After receiving the nucleotide sequences (see appendix 3), they were edited using MAFFT plugin in Geneious version 8.1.8 created by Biomatters (Kearse, *et al.*, 2012) and search done using online

Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information, USA.

Table 1. Information of primers used.

Target gene	Primer name	Primer sequence (5'-3')	Reference
Blood meal host identification			
12S Ribosomal RNA gene	12S3F	GGGATTAGATACCCCACTATGC	(Roca, *et al.*, 2004)
	12S5R	TGCTTACCATGTTACGACTT	
Arbovirus identification			
Flavivirus	Fu1	TACAACATGATGGGAAAGAGAGAGAA	(Eastwood, *et al.*, 2020)
	CDF2	GTGTCCCAGCCGGCGGTGTCATCAGC	
Alphavirus	Vir2052F	TGG CGCTATGATGAAATCTGGAAT GTT	(Eastwood, *et al.*, 2020)
	Vir2052R	TACGATGTTGTCGTCGCC GAT GAA	
Pan Phlebovirus	Ph_F1	TCAARAAGAMICAACATGGTGG	(Ohlendorf, *et al.*, 2019)
	Ph_R1	TATGCCYTGCATCATYCCWG	
	Ph_F2	GGACTTAGAGAGATYTAYGTITTGG	
	Ph_R2	ACATGRTGACCYTGRTTCCA	

4.0 RESULTS

4.1 Blood meal host identification

Of the 328 blood fed mosquito samples processed, 279 (85.1%) was successful. This number was arrived at owing to the method used in analysis. We sequenced all the samples to obtain blood meal host identity and because this is quite an expensive exercise, the number therefore was justified in accordance with the project's budget. Most blood meals were from tree hyrax (*Dendrohyrax dorsalis*) (70.6%, 197/279), followed by suni/antelope (*Neotragus moschatus*) (16.1%,45/279) and human (*Homo sapiens*) (8.6%, 24/279). Other avian and mammalian hosts represented in blood meals were *Procavia capensis* (rock rabbit; 1.1%), *Xiphiorhynchus guttatus* (woodcreeper bird; 1.1%), *Myotis spp (*mouse-eared bat; 0.72%), *Tragelaphus scriptus* (bushbuck; 0.36%), *Tapirus indicus* (Malayan tapir; 0.36%), *Myemeciza exsul* (bird species, 0.36%), *Crocuta crocuta* (spotted hyena; 0.36%) and *Chiroderma villosum* (hairy big-eyed bat; 0.36%) (Figure 2 & Table 2)

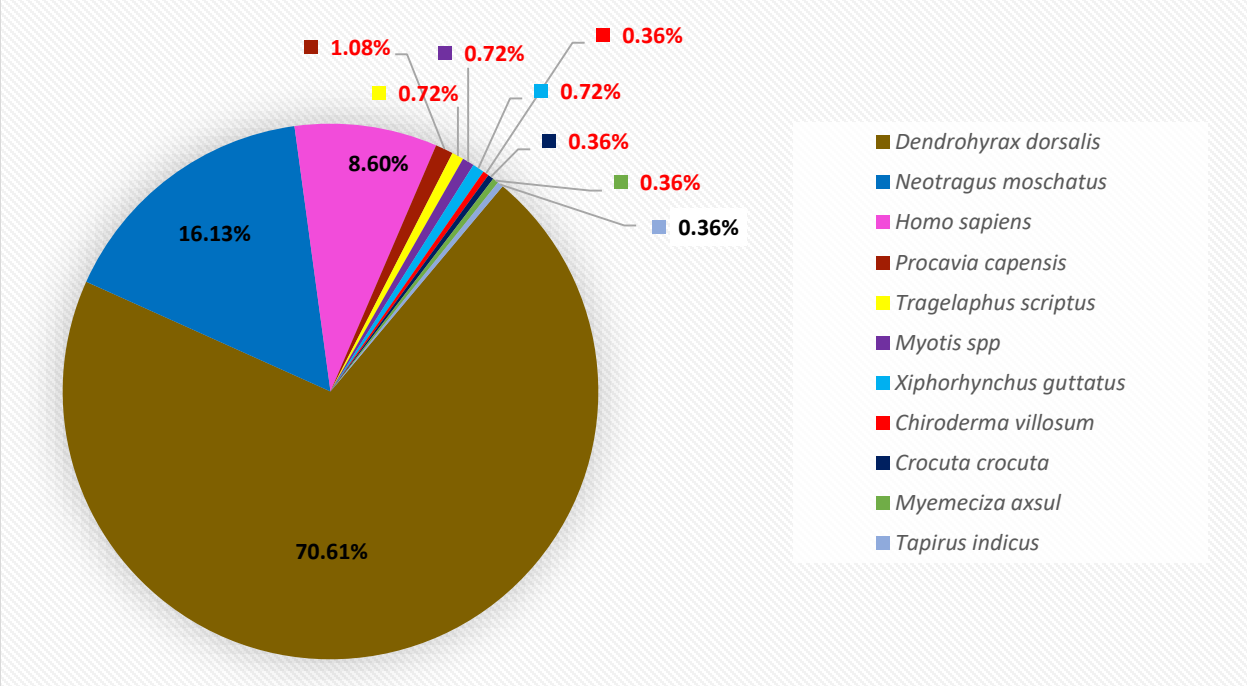

Figure 2. Host blood representation

4.2 Virus screening

In total, 11974 mosquitoes (unfed) organized into 500 pools and further 50 super pools, were processed for virus culture. This number represented more than three-quarters of the total samples collected. Since we employed both molecular and cell culture for virus screening, we selected the high number to increase the chances of obtaining any positive result. It was also dictated by the treatment, day and time of collection and yearly distribution. Two super pools tested positive with reproducible CPE observed between days 5-7 post inoculation. RNA extraction, RT-PCR, then sequencing conducted on the supernatant of the culture-positive samples, identified the isolates as WNV. Sequence analysis of the identified isolates showed similarity to lineage I WNV strains with close relationship (98% identity) to a mosquito isolate described in Senegal (accession number KY703854.1), See appendix 4. However, there was no virus positive result from the fed samples from RT-PCR screening directly.

4.3 Phylogenetic re-construction

Nucleotide sequences were edited in Geneious version 8.1.8 created by Biomatters (Kearse, *et al.*, 2012) using MAFFT plugin for nucleotide alignment. Phylogenetic tree was constructed using Mega 7 software. Briefly, analysis was done by Maximum Likelihood method The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura, *et al.*, 1993). The tree with the highest log likelihood (-568.71) 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-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis

involved 46 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were 197 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar, *et al.*, 2016).

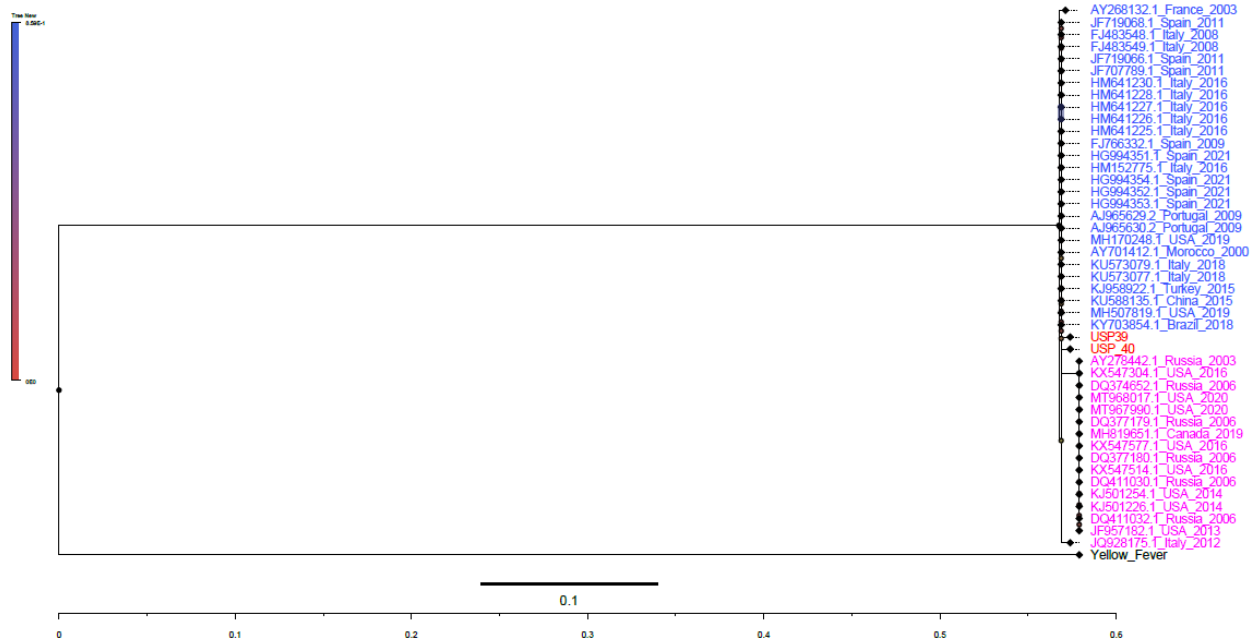

Figure 3. Phylogenetic Tree: Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura, *et al.*, 1993).

5.0 DISCUSSION

We found that *Ae. chaussieri* feeds on a wide range of vertebrate hosts but predominantly on tree hyrax. The apparent preference for tree hyrax could be attributed to several reasons. First, it could be that tree hyrax is the predominant species among the order Rodentia that comprises the majority of small mammals in Oloolua Forest (Meroka, Ben N, Gichuki, *et al.*, 2018). This would therefore lead to high rate of interaction between tree hyrax (host) and mosquitoes (vector) in the area. Other reported vertebrate hosts in the area includes vervet and colobus monkeys, baboons, duikers, bush pigs, water buck, and hyenas (Tchouassi, *et al.*, 2019a). However, these animals were not represented in the blood meals, suggesting proclivity in host feeding pattern of this mosquito. Second, a shared ecological niche between the mosquito and the tree hyrax linked to the biology of the mosquito could be at play. Tree hyraxes spend most of their time on trees and may be exposed to this mosquito that likely breeds or lay eggs in tree holes, hence frequent contact that might lead to high biting rate. However, primate species in the forest also spend much of their time especially at night on top of trees yet they were not recorded among observed blood meal sources. The data represents the first report of the feeding habits in this mosquito, an important biologic feature that contributes to the vectorial capacity of a disease vector.

Data from blood meals, show that *Ae. chaussieri* also feeds on humans. This ability to feed on diverse wildlife and humans, suggests the mosquito could serve as a potential bridge vector for zoonotic pathogens. Similar mechanism has been described for other *Aedes* species including *Aedes aegypti* which transmits zoonotic viruses such as chikungunya virus from monkeys to humans (Turell, *et al.*, 1992).

A strain of WNV was isolated and detected in two mosquito super pools, being the second reported case for any *Aedes* mosquito in Kenya after *Aedes sudenensis* from Garissa during 2007/8 RVF outbreak (Crabtree, *et al.*, 2009). The finding expands the association of mosquitoes with this virus. Previously, WNV has been reported in the *Aedes* mosquito *Aedes vexans* in the USA in 1999 (Sardelis, *et al.*, 2001). Globally, WNV has been detected and/or isolated in *Culex* mosquitoes largely incriminated as primary vectors of the virus. Examples include *Cx. univittatus, Cx. pipiens, Cx. antennatus, Cx. quinquefaciatus, Cx. tarsalis, Cx. triataenorhynchus, Cx. modestus, Cx. vishnui* in Africa, Europe, America and Asia (David, *et al.*, 2016; Sardelis, *et al.*, 2001; Sule, *et al.*, 2018). In Kenya, WNV has been isolated or detected in diverse *Culex* mosquito species including *Cx. univittatus* (LaBeaud *et al.*, 2011; Ochieng *et al*., 2013). The species *Cx. quinquefaciatus, Cx. vansomereni* and *Cx. univittatus* were found to be competent WNV vectors based on laboratory experimental infection assays (Lutomiah, *et al.*, 2011). The detection/isolation of WNV in diverse mosquito species other than the widely known *Culex,* points to a broader vectorial range of the virus than previously known. Perhaps, this could explain why WNV is currently the most widespread flavivirus worldwide (Fall, *et al.*, 2016). Nonetheless, additional studies to elucidate the competence of *Ae. chaussieri* to WNV are required.

The transmission cycle of WNV involves mosquito vectors and certain bird species that serve as reservoir host of the virus (Nyamwaya, *et al.*, 2019). Outbreaks of WN fever occurs in humans and horses considered as dead-end hosts (Fall, *et al.*, 2021; Sule, *et al.*, 2018). Thus, only birds can develop viremia sufficient to infect mosquitoes. Coincidentally, the blood-feeding host profile of *Ae. chaussieri* included wild bird species, which could be a source of virus infection of the mosquitoes. However, this cannot be verified from the present data given absence of detections

from any blood-fed mosquito that could be as a result of too low virus titer to be picked by the primers used.

The detected WNV grouped with previous strains in lineage 1 and closely related to an isolate from West Africa. The finding is consistent with previous studies identifying this lineage among mosquitoes in Africa (Jimenez-Clavero, *et al.*, 2006; May, *et al.*, 2011; Venter, *et al.*, 2011). WNV lineage 1 is considered the most widely distributed and associated with neuroinvasive diseases and major outbreaks globally (Fall, *et al.*, 2014; Murray, *et al.*, 2010; Petersen, *et al.*, 2001). Spread of this virus lineage could be facilitated by bird migratory activities (Ain-najwa, *et al.*, 2020; Rappole, *et al.*, 2003). The virus lineage could be widespread in circulation in Kenya following detections in arthropod vectors such as mosquitoes (LaBeaud, *et al.*, 2011) and ticks (Lwande, *et al.*, 2013).

Mainly single hosts were represented in individual mosquito meals with no evidence of mixed-hosts feeding. This trend may have been affected by use of PCR-Sanger sequencing of a single gene marker that has been found to poorly resolve multiple host meals from individual engorged specimens (Agha, *et al.*, 2019; Estrada-Franco, *et al.*, 2020; Tchouassi, *et al.*, 2021). More advanced techniques capable of circumventing this gap including next-generation sequencing (Muturi, *et al.*, 2021) can be explored to characterize host-vector interactions.

CONCLUSION

This study provides the first report of association of WNV with *Ae. chaussieri* with feeding habits mostly on wildlife predominantly on tree hyrax. The detection/isolation in this mosquito extends the spectrum of potential vectors of WNV. Its vectoring role as bridge vector of the virus could be facilitated by host feeding on humans as well. Further elucidation of its vectorial capacity including vector competence for viruses is recommended. From the blood meal analysis data, it was evident that *Aedes chaussieri* fed more frequently on tree hyrax. However, with the absence of suitable method to determine multiple host feeding behavior of an individual mosquito, this could not be ascertained to be true. Therefore, further studies to determine multiple host feeding would be recommended.

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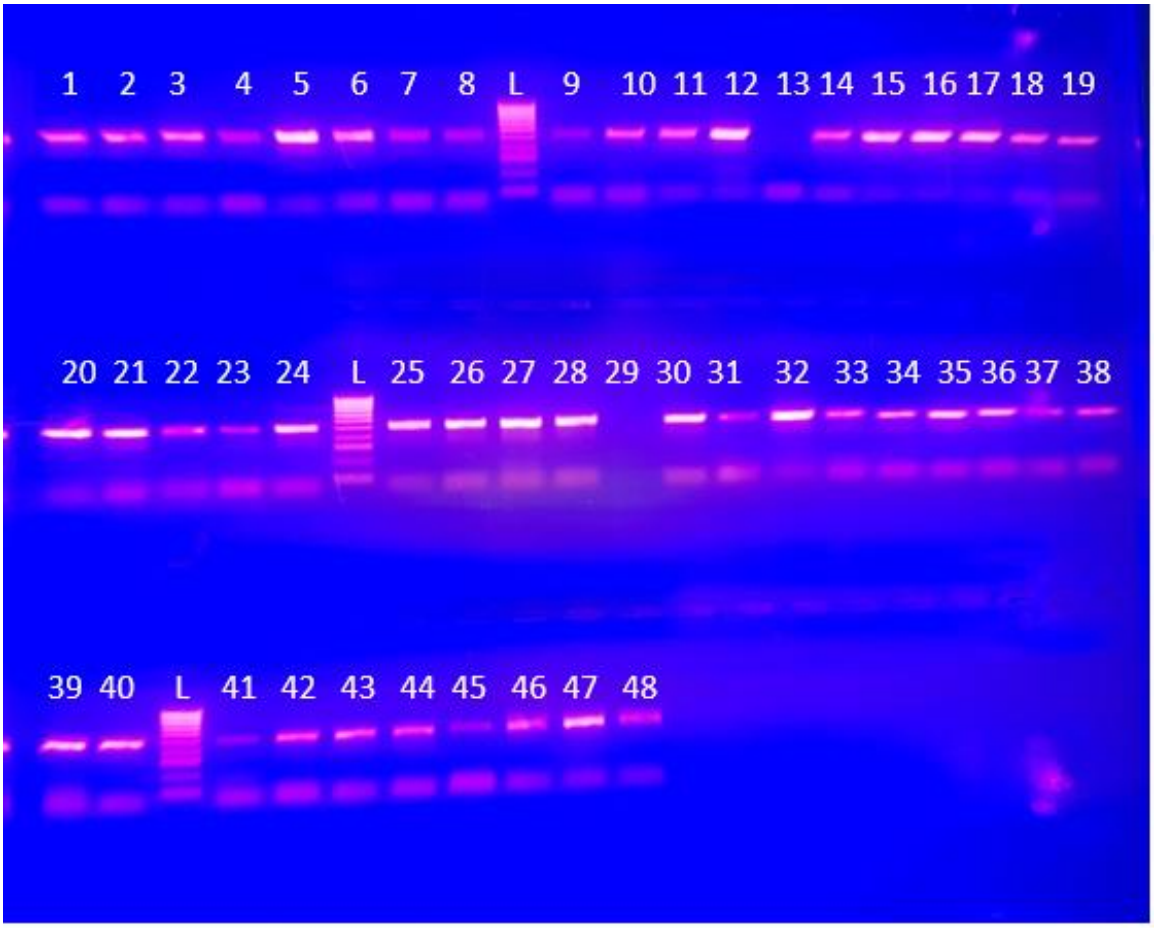
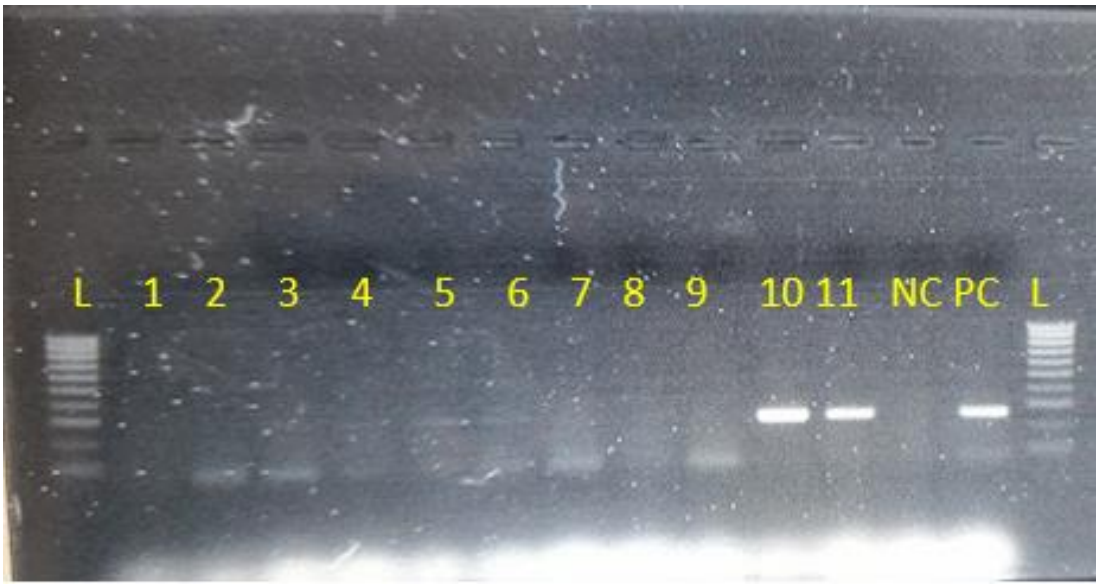
APPENDICES



Appendix 1. Gel electrophoresis image of samples of bloodmeal analysis using 12S primers

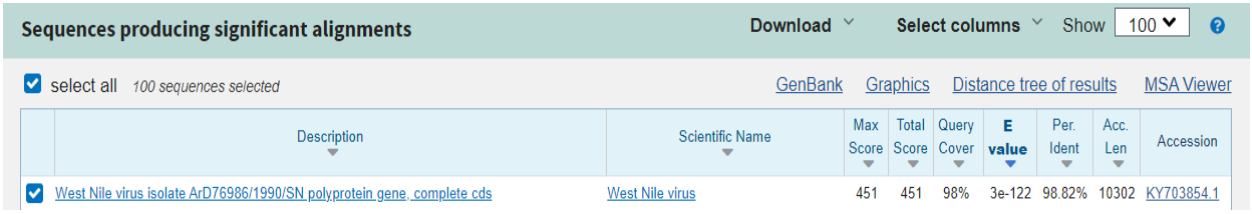


Appendix 2. Gel electrophoresis image of samples of screened for viruses using Flavivirus primers

Sample	Sequence
USP 39	TTTTACAACCTGTATGGGAAAGAGAGAGAAAAAACCCGGAGAGTTCGGAAAGGCCA AGGGAAGCAGAGCCATATGGTTCATGTGGCTCGGAGCTCGCTTTCTGGAGTTCGAAG CTCTGGGCTTTCTCAATGAAGATCACTGGCTTGGAAGAAAGAACTCAGGAGGAGGT GTCGAGGGCTTGGGCCTCCAAAAACTGGGTTACATCCTGCGTGAAGTTGGCACCCGA CCTGGGGGCAAGATCTATGCTGATGACACCGCC
USP 40	AAAACCCGGAGAGTTCGGAAAGGCCAAGGGAAGCAGAGCCATATGGTTCATGTGGC TCGGAGCTCGCTTTCTGGAGTTCGAAGCTCTGGGCTTTCTCAATGAAGATCACTGGC TTGGAAGAAAGAACTCAGGAGGAGGTGTCGAGGGCTTGGGCCTCCAAAAACTGGGT

	TAC
Positive Control	TTACAACATGATGGGAAAGAGAGAGAAAAAACCCGGAGAGTTCGGAAAGGCCAAG GGAAGCAGAGCCATATGGTTCATGTGGCTCGGAGCTCGCTTTCTGGAGTTCGAAGCT CTGGGCTTTCTCAATGAAGATCACTGGCTTGGAAGAAAGAACTCAGGAGGAGGTGT CGAGGGCTTAGGCCTCCAAAAACTGGGTTACATCCTGCGTGAAGTCGGCACCCGAC CTGGGGGCAAGATCTATGCTGATGACACCGCCGGCTGGGACACT

Appendix 3. USP sample sequence.



Appendix 4. Portion of the Basic Local Alignment Search Tool (BLAST) showing the similarity index of the resulting sequence.