EVALUATION OF LAMU AND TRANS NZOIA POPULATIONS OF *AEDES AEGYPTI* AS CHIKUNGUNYA VIRUS VECTORS UNDER TWO DIFFERENT INCUBATION TEMPERATURES

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university

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This thesis has been submitted for examination with our approval as University supervisors

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DEDICATION

I dedicate this work to my beloved grandmother the late Esther Mutio Mutisya the source of inspiration in my life since I was a small child.

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This thesis is not a product of one person. It developed into the planned products through the committed hands of several people who deserve special recognition.

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LIST OF ACRONYMS AND ABBREVIATIONS

CHIKV	Chikungunya virus
RNA	Ribonucleic acid
WHO	World Health Organization
DEET	N, N- Diethyl- meta- toluamide
DPI	Day Post Infection
EIP	Extrinsic incubation period
EIT	Extrinsic incubation temperature
MIB	Midgut infection barrier
MIR	Midgut infection rates
MEB	Midgut escape barrier
SGI	Salivary gland infection
KEMRI	Kenya Medical Research Institute
VHF	Viral Hemorrhagic Fever
CVR	Centre of Virus Research
L: D	Light: Day
MEM	Minimum Essential Media Eagles
FBS	Fetal bovine serum

CPE	Cytopathic effect
RH	Relative Humidity
BSL2	Biosafety Level 2
°C	Degree Centigrade
CO_2	Carbondioxide
SPSS	Statistical Package for the Social Sciences
ACUC	Animal Care and Use Committee
PPE	Personal Protective Equipment
ERC	Ethical Research Committee
BSC2	Biological Safety Cabinet 2
$NaHCO_3$	Sodium bicarbonate

ABSTRACT

The rate at which the chikungunya virus (CHIKV) infects and disseminates in *Aedes aegypti* can be influenced by temperature which affect the distribution and incidence of vector-borne diseases. The first chikungunya fever epidemic in Kenya occurred in Lamu Island in 2004/05. This study investigated the vector competence of *Ae. aegypti* for CHIKV from Lamu and Tans nzoia areas and the influence of varying Extrinsic Incubation Temperature (EIT) of 32°C and 26°C that resembles those in the two regions. Four-day old mosquitoes were exposed to CHIKV through a membrane feeding. They were then incubated in temperatures mimicking the mean annual temperatures for Trans-Nzoia (26°C) and Lamu (32°C). The midgut infection rates (MIR) of *Ae. aegypti* sampled from Lamu region was significantly (p=0.0037) higher than those sampled from Trans nzoia with statistically significant difference observed for the coastal *Ae. aegypti* at EIT 26°C and at 32°C. The MIR of *Ae. aegypti* from Trans nzoia was significantly (p=0.0004) affected by the EIT, with mosquito reared at EIT 32°C exhibiting higher MIR than those reared at EIT 26°C. There was a significant (p=0.0331) interactive effects of the region, EIT and DPI on MIR. The DIR-L and DIR-H was higher in mosquitoes sampled from Lamu regardless of the EIT while those from Trans nzoia, dissemination rates were significantly higher at higher EIT of 32°C. These findings show a clear difference between the two mosquito populations in their ability to disseminate infection with influence in high temperature enhancing vector competence. This could explain the distribution and spread of CHIKV into susceptible host. Such information is insightful and may assist the public health sector to focus surveillance and initiate vector control strategies and responses when there is a potential threat in prone areas.

CHAPTER ONE

INTRODUCTION

1.1 Chikungunya fever

Chikungunya fever is one of the most important mosquito-borne viral diseases in countries in Africa. The name Chikungunya, which originates from Makonde, a language spoken in Tanzania where it was first isolated means "that which bends up" or walking bent over", referring to the posture patients assume with the resulting arthralgia (Kondekar & Gogtay, 2006). The disease is caused by Chikungunya virus (CHIKV) an *alphavirus* belonging to the family *Togaviridae*, which was first isolated in Africa during dengue like illness in 1952-1953 (Ross, 1956). The debilitating disease is characterized by fever, skin rash, and arthritis-like pain in small peripheral joints that last for weeks or months (Chhabra *et al.,* 2008). There is no vaccine or treatment for CHIKV infection (Couderc *et al*., 2009). Therapy is therefore based on the patient's symptoms where antipyretics and anti-inflammatory agents are the most appropriate treatments for the primary signs. In addition to this drug therapy, bed rest and fluids intake are recommended (Tesh, 1982). In Africa, the virus has a sylvatic cycle involving wild primates and forest *Aedes* mosquitoes, while in Asia and the Indian Ocean it circulates exclusively between humans and *Aedes* spp. mosquitoes (Jupp and McIntosh, 1988). The principal vectors of CHIKV in the Indian Ocean basin are *Aedes aegypti* and *Aedes albopictus* (Reiter *et al*., 2006) which have been implicated in the CHIKV transmission cycle due to vector competence (Dubrulle *et al.,* 2009) and virus isolation from field-collected mosquitoes (Sang *et al.,* 2008; Leo *et al.,* 2009).

1.1.1 Transmission cycle of CHIKV and risk factors

Chikungunya virus is enzootic across tropical regions of Africa and Asia while in West and Central Africa it is believed to be maintained in a sylvatic cycle involving wild non-human primates and forest-dwelling *Aedes spp*. mosquitoes. The virus has been isolated

from sylvatic mosquito species in several countries including Senegal, Central African Republic and South Africa (Powers & Logue, 2007). The mosquito species involved vary geographically and with ecological conditions. Chikungunya virus is transmitted to humans from infected non-human primates and other humans by the bite of *Aedes aegypti* mosquitoes. This mosquito thrives in urban areas with favorable conditions for breeding, presence of water storage in containers, discarded water holding containers and other areas where stagnant water can accumulate. Evidence exists that CHIKV can also be passed from an infected mother to a developing fetus (Weaver, 2006; Chhabra *et al.,* 2008). Furthermore, inhalation of aerosolized CHIKV in a laboratory setting may lead to CHIKV infection (Scherer *et al.,* 1980).

1.1.2 Effect of temperature on virus infection and dissemination

The development, survival and arbovirus replication in mosquito vectors is greatly influenced by temperature which alters the length of the extrinsic incubation period (EIP), the time between ingestion of an infectious blood meal and when transmission to a subsequent host is possible (Chamberlain & Sudia, 1955; Patz *et al.,* 1996). High extrinsic incubation temperature (EIT) is also known to affect vector competence rates. For example, in one study, infection rates were found to be much higher in F_1 adult *Aedes epactius* mosquitoes reared at 18°C than in those reared at 27°C in a study conducted involving St. Louis encephalitis virus (Hardy *et al.,* 1980).

Extrinsic incubation temperature can affect infection, dissemination, and transmission rates for numerous arboviruses, including West Nile virus (WNV) (Takahashi 1976, Turell *et al.,* 1985, Watts *et al.,* 1987, Hardy, 1989, Turell 1989). Studies have indicated that there is a direct correlation between the ability of mosquitoes to transmit arboviruses while feeding and the EIT (Chamberlain & Sudia, 1961); transmission rates increase as the EIT increases from 18°C to 30°C (Hardy *et al.,* 1983). It has been demonstrated that high EIT shortened the extrinsic incubation period for WNV in *Culex uvivittatus* Theobald (Cornel *et al.,* 1993). In general alphaviruses appear to require a shorter EIP of 2-9 days.

1.1.3 Signs and Symptoms of chikungunya fever

Chikungunya virus is traditionally considered to be benign and non-life threatening. Its incubation period typically ranges from 2-4 days. Viremia lasts 2-10 days (Robinson, 1955; Kam *et al.,* 2009) and is higher in newborns and adults above the age of 60 (Laurent *et al.,* 2007). The classic triad symptoms are fever, arthralgia and rash distributed on the trunk, limbs and face. Onset of fever is usually acute and is associated with chills and rigor (Queyriaux *et al.,* 2008; Sissoko *et al.,* 2008). It precedes the rash and joint pain and usually reaches as high as 40°C and can remit after 4-5 days resulting in a "saddleback" fever (Powers & Logue, 2007).

1.1.4 Diagnosis of chikungunya fever

Due to the absence of commercial vaccines and specific treatment for CHIKV, early detection and diagnosis is imperative for control of infection. Clinically, CHIKV infection can be confused with dengue virus (DENV) infection due to the similar presentation, geographical distribution and vectors (Carey, 1971). Thus, CHIKV infection usually requires a diagnostic test.

Available diagnostic methods are reverse transcriptase-polymerase chain reaction (RT-PCR), culture isolation and serology (Thein *et al.,* 1992; Powers *et al.,* 2000; Ho *et al.,* 2010). RT-PCR is useful in the first week of the viraemic phase (Parola *et al.,* 2006). Isolation of CHIKV is considered the gold standard of strain identification (Pialoux *et al.,* 2007; Ho *et al.,* 2010). However, this is not commonly used in the clinical setting. Other methods include viral culture RT loop-mediated isothermal amplification, immunofluorescence assays and plaque reduction neutralization test (Litzba *et al.,* 2008; Lakshmi *et al.,* 2008).

1.1.5 Treatment of chikungunya fever

Treatment for Chikungunya fever is limited to supportive care: analgesics, antipyretics, rest and fluids intake (Simon *et al.,* 2008; Mahendradas *et al.,* 2008; Briolant *et al.,* 2004). There is no targeted antiviral treatment for CHIKV infection and treatment is mainly supportive. Thus, an anti-viral treatment is most useful for prophylaxis in vulnerable groups such as the immunocompromised and for management of severe cases (Po-Ying *et al.,* 2010).

1.2 Statement of the problem

Chikungunya virus is an important global re-emerging mosquito-borne *alphavirus* transmitted principally by *Aedes aegypti* mosquitoes which is well distributed in most parts of Kenya, and is generally highly anthropophilic (Harrington *et al.,* 2001) and the risk of CHIKV is therefore likely to be widespread. The first significant chikungunya fever outbreaks were first detected in Newala District of Tanzania in 1952- 1953 (Ross, 1956) around the Lake Victoria basin and spilled over to Kenya (Robinson, 1955). CHIKV re-emerged in Kenya in 2004 on Lamu Island (Sergon *et al.,* 2008), and later spread to other Indian Ocean with low level transmission of the virus been documented around the Lake Victoria basin including Busia (Mease *et al.,* 2011). Despite CHIKV having been associated with major epidemics that cause significant morbidity, there is no vaccine against the virus or treatment for the disease (Couderc *et al.,* 2009). In May 2016 a chikungunya outbreak occurred in Northern Kenya, these outbreaks could be due to high temperatures in the area since the virus replicates fast in warm weather. Hence, this could suggest a potential significant role of temperature in CHIKV transmission. Therefore, assit the public health sector to initiate vector control strategies and responses in prone areas.

1.3 Justification

Water reservoirs serve as excellent breeding grounds for mosquitoes such as *Aedes aegypti* species while warm, humid climates are environmental conditions that favor mosquito survival. High population density, lack of adequate resources for vector control and hygiene adds to the vulnerability of humans to CHIKV infection. The unique molecular features of the recently analyzed Indian Ocean isolates of CHIKV (Padbidri and Gnaweswar, 1979) suggest that the virus can evolve rapidly. Although there is abundant information that *Ae. aegypti* is the principal vector of CHIKV, knowledge on the vector competence of different populations of this species for CHIKV is scanty. Extrinsic incubation temperature has been shown to influence the replication and dissemination of arboviruses in vectors (WHO, 2007). Mosquito susceptibility to arbovirus infection resides primarily in the midgut; this susceptibility can vary between mosquito species and geographical strains of the same species or between individuals of the same mosquito population (Watts *et al.,* 1973). Inspite of *Ae. aegypti* being well distributed in both areas this may suggest that extrinsic incubation temperature might have an influence on the replication and dissemination of CHIKV in *Ae. aegytpi* since Lamu experiences higher annual mean temperature compared to Trans Nzoia. Through the recent epidemics, CHIKV has demonstrated its ability to spread and infect large proportions of the populations, hence it is important to study the effect of environmental factor such as temperature on the susceptibility to, and transmission of CHIKV, by *Ae. aegypti* populations. This information is important in understanding the epidemiology of chikungunya fever.

1.4 Research Questions

1. What are the infection rates of CHIKV in Lamu and Trans nzoia *Ae. aegypti* populations' under varying extrinsic incubation temperature (EIT)?

2. What are the dissemination rates of CHIKV in Lamu and Trans nzoia *Ae. aegypti* populations' to the legs under varying EIT?
3. What are the dissemination rates of CHIKV in Lamu and Trans nzoia *Ae. aegypti* populations' to the heads under varying EIT?

1.5 Objectives

1.5.1 Main Objective

To evaluate the vector competence of Lamu and Trans Nzoia *Ae. aegypti* populations for CHIKV under varying temperature

1.5.2 Specific Objectives

1. To determine the infection rates of chikungunya virus in Lamu and Trans-Nzoia *Ae. aegypti* populations under varying extrinsic incubation temperature.
2. To determine the dissemination rates of chikungunya virus in Lamu and Trans nzoia *Ae. aegypti* populations' to the legs under varying extrinsic incubation temperature.
3. To determine the dissemination rates of chikungunya virus in Lamu and Trans nzoia *Ae. aegypti* populations' to the heads under varying extrinsic incubation temperature.

CHAPTER TWO

LITERATURE REVIEW

2.1 Classification of CHIKV

Chikungunya virus can be classified into encephalitic and arthritic viruses. They are six viruses that belong to the arthritic virus group and they include O' nyong-nyong virus, Semliki forest virus, Ross River virus, Sindbis virus, Mayaro virus and CHIKV (Strauss & Strauss, 1994). Examples of encephalitis viruses are Western equine and Venezuelan encephalitis viruses (Couderc & Lecuit, 2009). Chikungunya fever is one of the most important mosquito-borne viral diseases in countries in Africa and Southeast Asia, including India. The disease is caused by chikungunya virus (CHIKV) an alphavirus belonging to the family Togaviridae, which was first isolated in Africa during dengue like illness in 1952-1953 (Ross, 1956). The name Chikungunya originates from Makonde language in Tanzania where it was first isolated, and the name means "that which bends up" or walking bent over", referring to the posture patients assume with the resulting arthralgia (Kondekar & Gogtay, 2006).

2.2 Virus properties of CHIKV

Alphaviruses are small, icosahedral-shaped, enveloped viruses, with about 70nm in diameter (Mancini *et al.,* 2000). The alphavirus genome is a single stranded, positive-sense, RNA genome with an approximate 12,000 nucleotides long, it also has a subgenomic RNA encoding the structural proteins containing both a 5' cap and a poly (A) tail (Cancedda & Shatkin, 1979). The genome is about 11.8Kb and has 2 open reading frames (ORF), encoding a non-structural polyprotein and a structural polyprotein. The non-structural proteins are required for viral replication. The structural proteins are produced by translation of an mRNA that is generated from an internal, subgenomic promoter immediately downstream of the non-structural open reading frame. (Garoff & Simons, 1974; Ekstrom *et al.,* 1994; Garoff *et al.,* 1998). Three

phylogenetically distinct groups with distinct antigenic properties, based on differing partial CHIKV E1 protein sequences, have been identified namely the Asian phylopgroup, West African phylopgroup and East, Central and Southern Africa phylopgroup (Strauss & Strauss, 1994; Powers *et al.,* 2000).

2.3 Epidemiology of Chikungunya Virus

2.3.1 Reservoirs of Chikungunya Virus

Chikungunya virus can be both endemic and epidemic. It is maintained by two different transmission cycles, specifically sylvatic and human – mosquito – human (Powers *et al.,* 2000). In sylvatic cycles, the main reservoirs for CHIKV are non-human primates, rodents, birds and potentially other vertebrates (Diallo *et al.,* 1999; McCrae *et al.,* 1971). These cycles could lead to maintaining the virus in an endemic region while the simian populations are immunologically protected. In areas where CHIKV is endemic, there is usually a range of vectors, reservoirs and a local population with high herd immunity probably due to numerous and continuous transmission (Diallo *et al.,* 1999).

2.3.2 Vectors of Chikungunya virus

In most epidemics, there are usually just one or two vectors, namely *Ae. aegypti* and *Ae. albopictus*. The vectors of CHIKV in the Indian Ocean basin are *Aedes aegypti* and *Aedes albopictus* (Michael *et al.,* 2007) which are implicated in the CHIKV transmission cycle due to vector competence (Dubrulle *et al.,* 2009). These mosquitoes are opportunistic feeders but prefer to feed on humans (Valerio *et al.,* 2010). *Aedes aegypti* mosquitoes are the principal vector of CHIKV and are involved virtually in all chikungunya epidemics reported so far in Africa, India and other countries in Southeast Asia. Several attributes make *Aedes aegypti* an efficient vector for the CHIKV: it is highly susceptible to the virus, prefers to live close to people, seeks a blood meal during the daytime and bites almost painlessly-several people in a short period for one blood meal (Gibbons & Vaughn, 2002). The mosquito, well adapted to life in urban settings,

typically breeds in clean puddles of standing water and collections of water in artificial containers such as tin cans, pots, plastic containers, rain barrels, buckets and discarded tyres (Wilder-Smith & Schwartz, 2005).

2.3.3 Chikungunya Epidemics in Kenya

In 2004 however, epidemics occurred in Kenya and in 2004-2005, spread to the Comoros where it then subsequently spread to the Indian Ocean islands (Ligon, 2006; Powers & Logue, 2007). Chikungunya outbreaks in East Africa and the Indian Ocean have become more frequent and showed a distinct line of movement with most recent affecting the Kenyan Islands of Lamu in April to August 2004 and Mombasa in November to December 2004, and subsequently appeared in the Indian Ocean island of Comoros and Mauritius in January to May 2005. The World Health Organization estimated that more than 200,000 people had been infected in La Reunion alone, while in Comoros and Lamu the infections were estimated to be 215,000 (63% of the population) and 13,500 (75% of the population) respectively based on the serological surveys conducted (WHO, 2006; Sergon *et al.*, 2007; 2008).

Although the original source and vector of CHIKV in the Lamu outbreak was not determined, widespread domestic water storage could have facilitated vector breeding and human contact. Infrequent water replenishment (expected because of drought) has been shown to increase domestic *Ae. aegypti* populations in coastal Kenya (Subra, 1983). Climatic effects, particularly elevated temperature, on virus development in vector mosquitoes also could have enhanced transmission efficiency (Watts *et al.,* 1987).

Western Kenya is known to be an endemic area for malaria with mosquito vectors for both malaria and CHIKV transmissions being prevalent (Korenromp *et al.,* 2003). A previous seroprevalence study done on the distribution of arboviral infection in different rural regions in Kenya; CHIKV infection was found to be more prevalent in Busia. This seropositivity suggested that the predominant cause for this might have been due to previous exposure to infected mosquitoes during one or more outbreaks (Mease *et al.,*

2011). A most recent seroprevalence study done on pyretic children in Busia County in Western region showed the prevalence rate of CHIKV being 11.5% (Mwongula *et al.,* 2013).

2.4 Effect of Climate Change on transmission of Chikungunya virus

Heavy rains can create floodplains where mosquitoes breed; with storms, floods and droughts creating conditions conducive for water-mosquito-borne diseases (Linthicum *et al*., 1999; Epstein, 1999; Relman *et al.,* 2008). Warm, humid climates and water reservoirs serve as an excellent breeding ground for the vector of the virus, *Aedes* mosquitoes. An increase in temperature, susceptibility of mosquitoes to CHIKV increases (Mourya *et al.,* 2004). With drastic increases in global travel resulting in increased volume and speed of traffic coupled with environmental change resulting in increased vector distribution, there have been numerous new and re-emerging diseases, creating new challenges for policy makers, researchers and modern medicine to surmount. It has also been noted that in primarily rural regions these outbreaks tend to be of a smaller scale and appear to be heavily dependent upon the sylvatic mosquito densities that increase with periods of heavy rainfall, this was experienced in Ahmadabad in western India in 2006 (Lumsden,1955). The unique molecular features of the recently analyzed Indian Ocean isolates of CHIKV suggest that the virus can evolve rapidly. Studies are in progress to confirm genomic structure and virulence of the recent CHIKV from India (Schuffenecker *et al.,* 2006). During the outbreaks reported in the Indian Ocean islands, many travelers from industrialized countries with temperate climates became infected with CHIKV and were still viremic on return to their native countries setting off local transmission (Hochedez *et al.,* 2006; Beltrame *et al.,* 2007; Nicoletti *et al.,* 2008).

2.5 Prevention and vector control of Chikungunya virus

Programmes that involve environmental management are highly effective in reducing and disease transmission rates of CHIKV. A review conducted showed that community-based vector control strategies in addition to habitat control (through biological and chemical means) could reduce the density of *Aedes aegypti* (Heintze *et al.,* 2006). The resultant decline in the vector-host cycle can reduce transmission of the vector with a consequent decline in the incidence of the disease. Evidence suggests that when communities actively take part in abolishing the breeding places of *Aedes aegypti* (destruction, alteration, disposal or recycling of domestic containers); the density of larvae is significantly reduced (WH0, 1997). Personal protection such as applying insect repellant to the exposed skin can keep out *Aedes aegypti,* a daytime biter. 30% DEET in insect repellants have shown to provide an average of 5 hours of complete protection against *Aedes aegypti* bites after single application on the exposed skin (Fradin & Day, 2002).

2.6 Vaccine Development

One of the preparations harvested from green monkey kidney cells (GMKC) was found to induce high levels of antibodies, it was protective against intracranial challenge with homologous virus, it produced no detectable viraemia and it resulted in good protection in monkeys after challenge with four strains of CHIKV (Harrison *et al.,* 1967). In the latest development of vaccine development, a live CHIKV vaccine was developed via 18 serial passages through MRC-5 cell cultures by the U.S. Army (Levitt *et al.,* 1986). This attenuated vaccine, specifically 181/clone 25 (181/25) is immunogenic in humans but phase II safety trials have resulted in around 8.5% participants developing transient arthralgia (Edelman *et al.,* 2000). There is potential reversion due to the inherent instability of RNA genomes coupled with little attenuating mutations and further work was halted. There are new strategies implemented in the development of a CHIKV vaccine including the development of DNA chimeric vaccines and epitope based vaccines (Wang *et al.,* 2008). Development of vaccines which can induce similar lasting

protective immunity has been ongoing through the years however many trials have been stopped due to limited resources and lack of commercial potential (Kam *et al.,* 2009).

2.7 Vector competence of Aedes mosquitoes

Vector competence refers to the intrinsic permissiveness of an arthropod vector to infection, replication, and transmission of a pathogen (Woodring *et al*., 1996). It is also the capacity of an arthropod to acquire an infection and transmit it to a subsequent host, which can greatly vary among individuals and between populations (Turell *et al*., 1992). Urban or large outbreaks during 2004–2005 in East Africa and Comoros were also associated with the presence of *Ae. aegypti* mosquitoes. In addition to various field data, numerous laboratory studies have examined distinct populations of *Ae. Aegypti* to understand the variable susceptibilities of these species in the transmission of CHIKV (Soekiman, 1987; Banerjee *et al.,* 1988; Mourya *et al.,* 1994).

Understanding mosquito population/virus infection dynamics will be important as CHIKV continues to spread into new areas. In addition to *Ae. aegypti*, other common peridomestic species, including *Ae. Albopictus* and *Ae. Vittatus* have been found in abundance in CHIKV endemic areas and have been assessed for their vectorial capacity (Soekiman *et al.,* 1986; Mourya, 1987; Mourya & Banerjee, 1987; Turell *et al.,* 1992; Yadav *et al.,* 2003). Since all these three species were found to be competent vectors in the laboratory, their role as primary vectors in urban outbreaks of CHIKV disease should be considered (Reiter *et al.,* 2006).

2.8 Acquisition of arbovirus by the mosquito

2.8.1 Ingestion of virus

Establishment of an arbovirus infection in a mosquito following ingestion of virus is dependent on the amount of virus ingested by the mosquito and the susceptibility of the mosquito to infection by the virus. A blood meal goes directly into the posterior end of the midgut where a peritrophic membrane secreted by the epithelium, the blood is

digested (Chamberlain *et al.*, 1954; Thomas 1963; Clements, 1963). Although mosquito susceptibility to arbovirus infection resides primarily in the midgut, susceptibility between mosquito species and geographical strains of the same species can vary widely between individuals of the same mosquito population (Chamberlain *et al.,* 1959; Watts *et al.,* 1973) and these variations can be explained by observed seasonal, regional and yearly differences in arboviral prevalence and the character of epidemics.

Any mosquito can acquire a virus when it ingests the infected blood of the host but failure of a mosquito to become infected following ingestion of an infectious blood meal can be attributed to the ingested blood having a virus concentration below the infection threshold, which is called midgut barrier which can be attributed to impermeability of the peritrophic membrane, variations in the permeability of gut cell membranes and limited number of specific virus receptor sites on the gut cell (Chamberlain & Sudia, 1961). If the PM forms a barrier to the passage of virus from the blood mass in the midgut of the mosquito to the epithelial cells, then the beginning of PM secretion will be of importance to the fate of the virus in the mosquito.

The time at which the secretion of the PM begins following a blood meal is about 0.5 hour or less for *Ae. aegypti* (Bertram & Bird, 1961; Freyvogel & Staeubli 1965). The length of time following the blood meal in which the PM does not form a potentially dense barrier to the passage of virus may vary in different mosquito species. This time seems to be less than 30 minutes in some species and longer in others. It is during this time that the PM is secreted and the virus particles could pass with minimum hindrance from surface layers of the blood mass to the posterior midgut (McLintock, 1978). In addition to the PM, the cell membranes of the midgut epithelium also form potential barriers to the passage of virus into and out of the epithelial cells. Studies have described that *Ae. aegypti* midgut epithelium has cell membranes with each of the cell epithelium being surrounded by a triple-layered membrane (Bertram & Bird, 1961).

During ingestion and digestion, the basal lamina expands in all directions and its layers stretch mainly in the longitudinal axis of the posterior midgut. It consequently could limit the expansion of the midgut during engorgement, and it has also been suggested that it functions like a membrane filter that could facilitate the transport of ions and macromolecules between midgut and hemolymph and prevent the passage of larger macromolecules, but the cell membranes collectively might also form a barrier to passage of viruses. Following ingestion of an arbovirus by a mosquito, where the infecting dosage surpassed the infection threshold, the course of the infection in the mosquito has been documented. There is an initial decrease in the quantity of virus below that ingested, followed by an increase to levels above those ingested (Houk *et al.*, 1980).

As incubation progresses the virus moves form one tissue to another; the salivary glands are among the last organs infected. The level of infection reached in the salivary glands is higher than in other organs. By the time the virus has reached the salivary glands, the virus titer in other organs has started to decrease and eventually disappears (Chamberlain & Sudia, 1961). Rate movement of the virus to the salivary glands and its replication in the glands are temperature dependent and contribute to variations in the extrinsic incubation period (Hardy *et al.*, 1983; Mclean, 1955). In the passage of an ingested arbovirus through its mosquito host, the salivary glands are the ultimate destination of the virus within the mosquito. Evidence that the virus reaches the various organs of the mosquito body via the hemolymph has been documented (Chamberlain & Sudia, 1961). The salivary glands are a site of replication and storage of virus before its transmission in the saliva to another vertebrate host. These salivary glands are paired and an individual gland of each species consists of three lobes; in *Ae. aegypti* the lobes are branched with each branch having its on salivary duct (McLintock, 1978).

2.9 Virus transmission barriers in mosquitoes

Arboviruses infect the mosquito midgut following ingestion of a viremic blood, replicate, disseminate to the salivary glands, and emerge into saliva to be transmitted when the mosquito takes another blood meal. The midgut and salivary glands act as barriers to virus infection and escape (Hardy *et al.,* 1983). The transmission of the virus to the vertebrate host depends upon the secretion of infectious virions in the saliva of the vector. Estimation of the amount of infectious particles transmitted by the mosquito after a blood meal is crucial to understand transmission and pathogenesis (Aitken, 1977; Smith *et al.,* 2005). Mosquito must salivate during blood feeding as the saliva contains different substances counteracting the host hemostatic response preventing blood coagulation and enhancing vasodilatation (Ribeiro, 2000; Schneider & Higgs, 2008). However, components of saliva may differ from one species to another (Calvo *et al.,* 2009). It has also been reported that saliva is able to enhance viral infections (Schneider *et al.,* 2006).

Knowledge of the sequential infection and multiplication of an arbovirus in the organs of a competent mosquito vector is a prerequisite to understanding mechanisms associated with barriers in incompetent vectors (Chamberlain & Sudia, 1961; McLintock, 1978). Once ingested by its mosquito host, a virus must overcome several obstacles if it is to be transmitted to a subsequent host. First, the virus must establish a productive infection in the mosquito midgut by overcoming a midgut infection barrier (MIB) (Bosio *et al*., 2000). Following replication in the midgut epithelium, virus must overcome a midgut escape barrier (MEB) and replicate in other tissues. Ultimately, virus must then infect the salivary glands and be shed in the saliva for transmission to the next vertebrate host (Bosio *et al*., 1998).

The minimum time necessary for an arbovirus to infect, multiply, and escape from the mosquito mesenteron into the hemocoel is variable depending upon the type and dose of virus, mosquito species, and temperature of extrinsic incubation. It is evident that ingested virus must multiply in the mesenteron before infection of the salivary glands

can occur (Hardy *et al*., 1983). The virus in the hemolymph serves as the source of infection of salivary glands. Some mosquito species or individuals within a single mosquito species may be quite susceptible to infection by the oral route but are poor transmitters of virus (Chamberlain & Sudia, 1961). Thus, it's becoming increasingly evident that the vector competence of mosquitoes for arboviruses is quite complex and is associated with multiple barrier systems which is considered a component to failure to mosquito infection.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

Lamu was selected to represent the Coastal region which has been associated with chikungunya while Trans-Nzoia was selected to represent an area where chikungunya fever has not been documented. In Trans Nzoia County, mosquito egg samples were obtained from Kiminini and Sasuri village in Trans Nzoia County while Lamu town and Shela Village represented Lamu County. The mosquito egg collection for both regions was done in the month of April 2012.

3.1.1 Lamu County

Lamu County is situated in Kenya's former Coast Province, at a longitude of 040°S´E and a latitude of 02° 17´S, and is headquarters in Lamu town. The county covers a strip of northeastern coastal mainland and the Lamu Archipelago. It has a population of 101,539 (Kenya Census, 2009) and its land area is 6,167 km^2. Lamu has a tropical savannah climate with average annual temperatures ranging between 13.6°C and 40.9°C. The county receives annual precipitation ranging between 900mm and 1100mm, with the rainy season experienced between May and June. From July the environment gradually gets hotter and dryer until March/April when it reaches 40°C. Shela Village on Lamu Island is a tangle of narrow sandy lanes some smaller thatched dwellings and mosques. It is about 3.2 km south of Lamu Town.

3.1.2 Trans Nzoia County

Trans Nzoia has latitude of 1.0567° N, and a longitude of 34.9507° E and a temperature range between 10 and 27 °C with an annual precipitation ranging between 1000 and 1200 mm, with the wettest months being experienced between April and October. The elevation of Kitale is about 1900 m. It has area coverage of 2,495.5 Km^2. The mean

annual temperatures range from 15°C to 20°C with average rainfall amounts of 11,200 mm per annum. Part of the second highest mountain in Kenya, Mount Elgon, lies in this county.

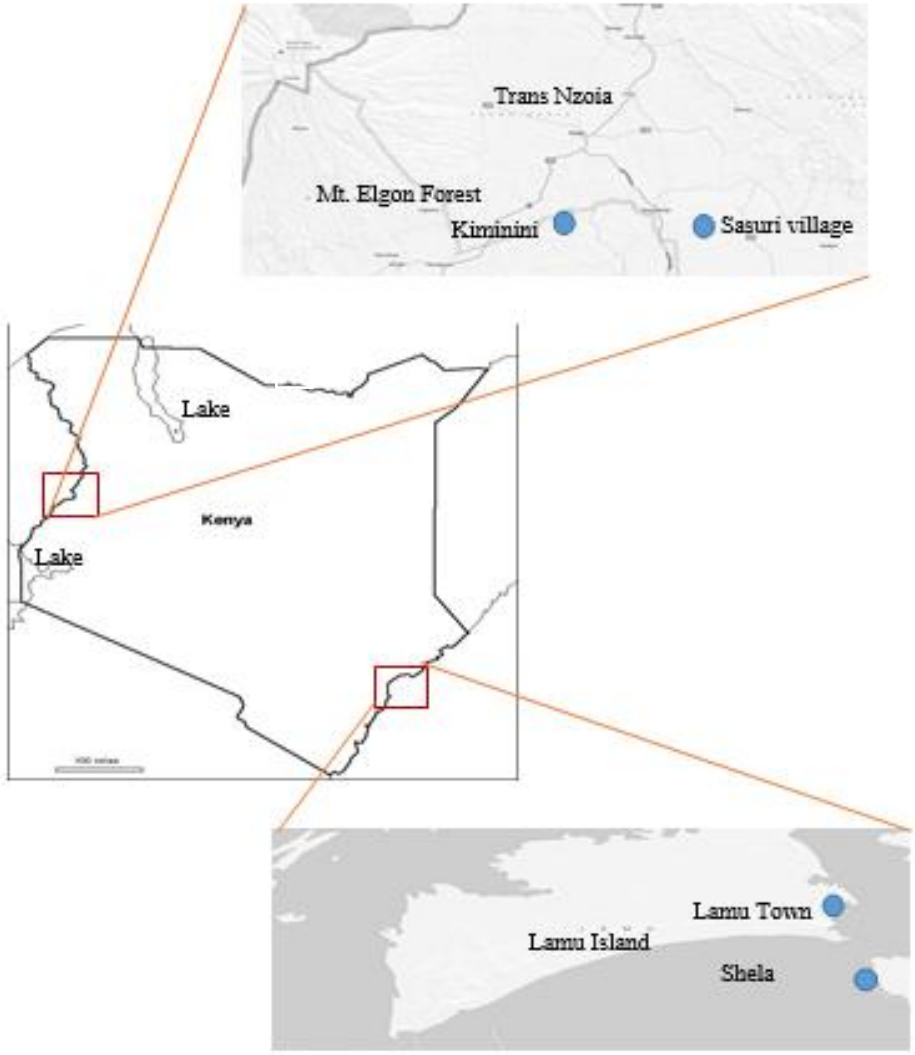

Figure 3.1: Map showing the study area and the sites where Aedes aegypti eggs were collected

3.2 Study Design

This was a cross-sectional field sampling, laboratory experimental study, with the temperature conditions of the respective regions within a laboratory setting.

3.3 Experimental Procedures

3.3.1 Collection of mosquito eggs

Eggs of *Ae*. *aegypti* were collected using oviposition traps that consisted of black plastic cups lined with oviposition paper and half-filled with water. A total of 25 ovitraps were placed at least 50 m apart in each of the study setting. These ovitraps with oviposition papers were left in the peridomestic areas for four days. On the 4th day, all the ovicups were collected and the eggs transported to the biosafety level-2 (BSL-2) laboratory where they were dried on damp cotton wool to quiescent state as earlier described (Steinly *et al.,* 1991; Chepkorir *et al.,* 2014) and stored in an air tight container at room temperature in the insectary.

3.3.2 Mosquito rearing

Mosquitoes were reared in the insectary, maintained at a temperature of 28 °C and 80 % relative humidity (RH), with a 12:12-h (Light: Dark) photoperiod. The eggs were hatched, and the larvae fed on TetraMin® fish food until pupation. Pupae were transferred to small plastic cups half-filled with water, placed in 4-l plastic cages screened with netting material on top and allowed to emerge into F0 adult mosquitoes. The emerging adults were then morphologically identified under a dissecting microscope using taxonomic keys of Edwards (Edwards, 1941), to confirm that only *Ae. aegypti* mosquitoes were used in the subsequent experiments. The identified female *Ae. aegypti* were returned to experimental cages, blood fed on clean laboratory-bred mice and provided with oviposition papers to lay F1 eggs. The F1 eggs were hatched and reared as outlined and only adult female mosquitoes were used in the succeeding experiments (Gerberg, 1970; Schwiebert, 2007).

3.3.3 Chikungunya Virus Propagation

CHIKV strain isolated from the 2004–2005 outbreak in Lamu Island (Lamu001) was used in this study, the East/ South/Central Africa and Indian Ocean genotype (group III), subgroup: East/South/Central African subgroup (IIIa and b) (Njenga *et al.,* 2008; Cui *et al.,* 2011). The working stock virus was prepared by propagating CHIKV in Vero cells in T25 culture flasks. The infected cells were incubated at 37 °C and 5 % CO2 and observed daily for cytopathic changes before the virus was harvested. The virus stock titer was determined [log10 7.2 plaque-forming units (pfu)/mL] by plaque assay and aliquoted in cryovials and stored in liquid nitrogen tanks until usage (Reiskind *et al.,* 2008). The CHIKV was propagated in mass culture of Vero cells in tissue culture flasks. For production of a fresh virus for mosquito infection, Vero cells were grown in T-75 cell culture flasks to confluence in Minimum Essential Medium (MEM) with 10% fetal bovine serum (FBS), 2% glutamine, 2% antibiotic/ antimycotic solution. The CHIKV (600µls) was inoculated into the confluent cells and incubated at 37°C for 45 minutes to allow virus adsorption, after which maintenance medium (MEM with 2%FBS, 2% glutamine, 2% antibiotic/ antimycotic) solution was added. The infected cells were incubated at 37°C and 5% CO_2 and observed daily for cytopathic effects (CPE). When CPE was observed, the virus was harvested and aliquoted in cryovials and stored at -80°C until usage (Reiskind *et al.,* 2008).

3.3.4 Mosquito Infection using membrane feeder

Four-day old mosquitoes were deprived of sucrose solution and water 24 hours prior to exposure to the infectious blood meal using a membrane feeding apparatus through mice skin. The mouse skin which was used only once (Novak *et al.,* 1991) was transferred to the insectary in a zip locks bags. A Hemotek membrane-feeding system (Discovery Workshops, Accrington, U.K.) which employed an electric heating element to maintain the temperature of the blood meal constant at 37°C. The model had three heaters with each heater in this case representing 1st, 2nd and 3rd replicates; being attached to a small aluminum plate to which the mouse skin was fixed the blood filled into the membrane holder and the "O" was used to attach the membrane. Once the assembled membrane unit was completely ready, the infectious blood meal was prepared by adding working virus (500µl) to defribrinated sheep blood (500µl). The blood-virus mixture was pipetted into each membrane unit which was screwed onto the heating chamber. It was attached to the heating unit with the temperature in this chamber being kept constant at 37°C and each of the three heaters placed on different mosquito cages each containing 200 *Aedes aegypti* mosquitoes depending on the mortality experienced prior to feeding, which were allowed to feed for 45 minutes (Hagen & Grunewald, 1990; Cosgrove *et al.,* 1994). The membrane feeding was done in the insectary BSL2, gloves and lab coat were worn. Fully engorged mosquitoes were selected, transferred to 4-liter plastic cages and maintained on 10% glucose solution (Chepkorir *et al.,* 2014).

3.3.5 Vector Competence and Transmission barrier determination

The vector competence was determined by detecting virus in the mosquito abdomen, legs and heads homogenates. Three replicates were used for each set of the experiment conducted. The abdomen was tested to determine the infection status and the legs were assayed to determine the dissemination status. If the virus was detected in its body and leg, the mosquito was considered to have a disseminated infection. However, if virus was detected in its body, but not legs the mosquito was considered to have a non-disseminated virus infection limited to its midgut (Turell *et al.,* 1992).

Female mosquitoes with no infection of the body show a midgut infection barrier (MIB), while females with an infected body and no dissemination to the legs show a midgut escape barrier (MEB). The virus titers in the body and legs as a quantitative measure of the ability of the virus to replicate in mosquito tissues (Bosio *et al.,* 2000).

3.3.6 Infected mosquito asssay

A third of the fed mosquitoes was randomly sampled on day 7, 10, 13 post exposure (pe) and was dissected into abdomen, head and legs (Diallo *et al.,* 1999; Vazeille *et al.,* 2007; Dubrulle *et al.,* 2009). The abdomens of each mosquito were individually triturated in 1 ml of Eagle's Minimum Essential Medium (MEM) (Sigma Aldrich) diluents and frozen at −80 °C until assayed for CHIKV by plaque assay on Vero cell monolayers. The dilutions were inoculated on confluent Vero cell monolayers in 12-well plates. Each plate was labeled with sample and dilution descriptions and for each virus stock serial dilutions (10^0, 10 −1, 10 −2, 10 −3, and 10−4) were made.

3.3.7 Chikungunya Virus Quantification

3.3.7.1 Seed Culture Plate with Vero cells.

Plaque assay was used in this study to quantify the amount of the CHIKV in each sample. Approximately 24 hours before beginning the titration protocol, Vero cells were grown in 12-well plates. The wells were seeded at a density of 2 x 10^5 Vero cells per well in 2 ml of growth medium; Minimum Essential Medium (MEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% antibiotic/antimycotic and 2% L-glutamine. The plates were gently rocked back and forth, then side to side to ensure the cells were evenly distributed on the plate surface. Once the plates were seeded, the cells were incubated in a humidified CO_2 incubator at 37°C overnight. Before use, the cells were visualized by light microscopy to confirm confluency and even cell distribution (Chepkorir *et al.,* 2014).

3.3.7.2 Preparation of viral serial dilutions of the samples.

Each plate was labeled with sample and dilution descriptions and for each virus stock serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) were made. Five 1.5 ml micro centrifuge tubes from 10^0 to 10^{-4} were appropriately labeled with the sample description. 900 µl of MM (Minimum Essential Medium (MEM, Sigma-Aldrich) supplemented with 2% heat-inactivated fetal bovine serum (FBS), 2% antibiotic/antimycotic and 2% L-glutamine). 100µl of the co-transfection supernatant was added to the first tube and vortexed. Serial dilutions were performed by transferring 100µl to the subsequent dilution, using a new pipette tip each time (Chepkorir *et al.*, 2014).

3.3.7.3 Infecting of the monolayer cells.

The plates were checked under a light microscope to confirm 70% confluency. The labeled plates were carefully discarded the GM (Minimum Essential Medium (MEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2% antibiotic/antimycotic and 2% L-glutamine) from the plates by gently tipping them over

a waste bucket containing 0.5% NaOCL (5ml of bleach and 95ml of distilled water). 100µl of the appropriate viral dilution was added to each of the plates to infect the cells and gently rock plate back and forth, then side to side to evenly distribute the virus. This was repeated to all the corresponding plates and dilutions. The plates were incubated and gently rocked every 15 minutes for 1 hour. During this incubation, methylcellulose overlay medium (1.25% Methylcellulose in MEM with 2% FBS) which had been prepared was warmed in the water bath at 37°C (Chepkorir *et al.,* 2014). 1 ml of methyl cellulose solution was added gently to each well, taking care not to dislodge any cells. When the methylcellulose had set, the plates were incubated at 37°C in a humidified 5% CO_2 for 3 days. Plaques were visible within 1 to 2 days. On the 3rd day, methylcellulose was removed from the wells using disposable plastic pipette in bio-safety cabinet. Plates were fixed by adding 1 ml of 10% formaldehyde in PBS (1/10 diluted Formalin with PBS) over the cell and incubated for 1 hours at room temperature in the safety cabinet with UV light on to fix the plate completely. Formaldehyde was removed with plastic pipette and discarded into the specific waste bottle for formaldehyde. The plates were then stained using 0.5% crystal violet as the staining solution (0.5 g of powder form Crystal Violet into 100mL of 99.5% Ethanol). Dye was discarded and plates washed gently with tap water. Plates were absorbed on paper and air-dried at room temperature (Chepkorir *et al.,* 2014).

The viral titer was a quantitative measurement of the biological activity of CHIKV and is expressed as plaque forming units (pfu) per ml. To calculate the viral titer, the numbers of well isolated plaques were counted. The following formula was used to determine the titer (pfu/ml) of your viral stock (Turell *et al.,* 1984).

$$\frac{\text{Number of plaques}}{\text{Dilution factor} \times \text{Volume of diluted virus added to the well}} = \text{Plaque Forming Unit/ml}$$

3.3.8 Ethical considerations

KEMRI Animal Care and Use Committee (ACUC), Scientific Steering Committee (SSC) and Ethical Review Committee (ERC) approved all the procedures used in this study. The guidelines were strictly adhered to during the research (APPENDIX VIII, IX, X).

3.3.9 Data management and analysis

The collected data were analyzed using SPSS version 20.0 software package. Differences in mosquito counts due to differences in treatments were analyzed using chi-square test. A logit model was utilized in showing the nominal main effects of the region, temperature, EIP and their interactions on the infection rates of CHIKV. MIR was computed as (Number of positive midgut infections divided by Total number of mosquitoes tested) ×100 % (Turell *et al.,* 1984; Lutomiah *et al.,* 2011) for day 7, 10 and 13 in the order. The midgut infection rate was defined as the percentage of mosquitoes tested that contained the virus in their abdomen/midgut and the dissemination rates in the legs and heads as the percentage of infected (abdomen/ midgut positive) mosquitoes that contained virus in their legs and/or heads respectively. The differences between these groups were deemed statistically significant at $P<0.05$.

CHAPTER FOUR

RESULTS

4.1 Midgut infection rates for Lamu and Trans Nzoia Ae. *aegypti* population

In Lamu region infection rates were measured in a total of 329 *Ae. Aegypti* female mosquitoes in three replicates (156 in low and 173 in high temperature regimes). At low temperatures 26°C, the number of midgut infections increased from day 7 (n=52, 17.9%) to day 13 (n=52, 26.9%) post infection. At high temperatures 32°C, the midgut infections increased from day 7 (n=57, 17.3%) to day 10 (n=57, 26%) but decreased at day 13 (n=59, 16.2%). In Trans Nzoia region a total of 302 *Ae. Aegypti* female mosquitoes in three replicates were measured (146 in low and 156 in high temperature regimes). At low temperatures 26°C the number of midgut infections increased from day 7 (n=48, 5.5%) to day 13 (n=50, 13.7%) with a decline on day 10 (n=48, 4.1%) recorded. At high temperatures 32°C, the number of midgut infections were high at day 7 (n=52, 22.4%) and decreased in both day 10 (n=52, 16.7%) and 13 (n=52, 16.7%). The MIRs at 7, 10 and 13 days' post infection (DPI) for CHIKV in Lamu and Trans Nzoia *Ae. aegypti* reared at EIT of 26°C and 32°C are shown in Table 4.1 with midgut infection rate vis-a-vis temperature ranges trends shown in Fig. 2.

Table 4.1: Midgut infection rates of 7, 10 and 13 DPI of CHIKV in Lamu and Trans Nzoia *Ae. aegypti* reared at EIT of 26°C and 32°C.

Region	No. of midgut tested (n)	EIT[a]	DPI[b]	DPI inf	No. of midgut infections		MIR[c] (%)
Coastal	156	26°C	7	52	28		17.9
			10	52	36	106	23.1
			13	52	42		26.9
	173	32°C	7	57	30		17.3
			10	57	45	103	26.0
			13	59	28		16.2
Western	146	26°C	7	48	8		5.5
			10	48	6	34	4.1
			13	50	20		13.7
	156	32°C	7	52	35		22.4
			10	52	26	87	16.7
			13	52	26		16.7

Within each population and temperature conditions, the number of tested mosquitoes is the same for all the days of sampling

[a]EIT =Extrinsic Incubation Temperature

[b]DPI =Days Post Infection and

[c]MIR =Midgut Infection Rate

DPI inf= Day Post Infections (number of mosquitoes picked i.e. a third of number of midgut tested)

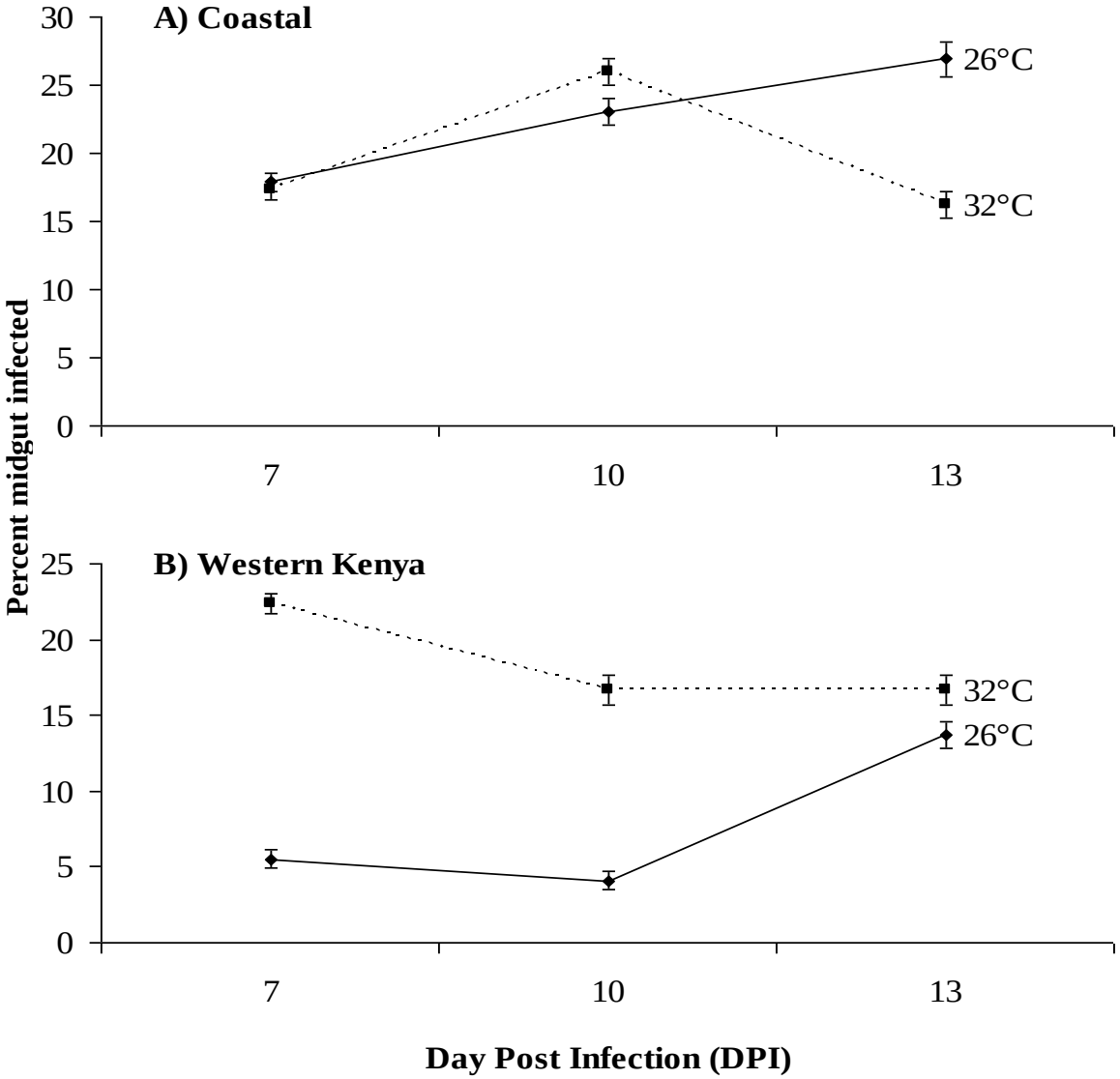

Figure 4.1: Proportion ± 95% confidence interval of Coastal-Lamu (a) and Western Kenya-Trans Nzoia (b) Ae. Aegypti infected at day 7, 10 and 13 post-infections at temperatures levels of 26°C and 32°C

4.2 Dissemination rates of CHIKV in Ae. *aegypti* populations from Lamu and Trans Nzoia

At low EIT 26°C, the disseminated infection rates in the legs (DIR-L) were highest on day 10 (n=36, 25.5%) and on day13 (n=42, 17.0%) among mosquitoes from Lamu region. At high EIT 32°C in the same region, the DIR-L increased from day 7 (n=30, 6.8%) to day 13 (n=28, 26.2%). At low EIT 26°C in the Trans Nzoia region, the disseminated infections in the legs were highest on day 7 (n=8, 20.6%) and declined on day 10 (n=6, 11.8%) but peaked on day 13 (n=20, 20.6%). At high EIT 32°C in the same region, the disseminated infections in the legs were highest on day 7 (n=35, 28.7%) and lowest on day 10 (n=26, 17.2%) but increased on day 13 (n=26, 26. 4%).

At low EIT 26°C temperatures, the disseminated infection rates in the head (DIR-H) were highest on day 7 (n=28, 10.4%) and lowest on both day 10 (n=36 2.8%) and day 13 (n=42, 2.8%). The number of DIR-H also increased from day 7 (n=30, 2.9%) to day 13 (n=28, 26.2%) in high EIT 32°C in the Lamu region. In Trans Nzoia region the DIR-H were highest on day 7 (n=8, 20.6%) and lowest on day 10 (n=6, 17.6%) at low EIT 26°C. At high EIT 32°C in the same region, the disseminated infections in the head were highest on day 7 (n=35, 25.3%) and lowest on day 10 (n=26, 8.0%) with an increase on day 13 (n=26, 23.0%). The dissemination rates of infected *Ae. aegypti* for CHIKV from the Lamu and Trans Nzoia at 7, 10 and 13 DPI and at low and high EIT (Table 4.2).

Table 4.2: Dissemination rates of CHIKV to the legs and head in Lamu and Western Kenya Ae aegypti reared at Extrinsic Incubation Temperature of 26°C and 32°C

				Number infected[(b)]		Dissemination rates (%)[(c)]	
Region	Number of midgut infections [(a)]	EIT	DPI (n)	Legs	Head	Legs	Head
Coastal	106	26°C	7	19	11	17.9	10.4
			10	27	3	25.5	2.8
			13	18	3	17.0	2.8
	103	32°C	7	7	3	6.8	2.9
			10	21	17	20.4	16.5
			13	27	27	26.2	26.2
Western Kenya	34	26°C	7	7	7	20.6	20.6
			10	4	6	11.8	17.6
			13	7	7	20.6	20.6
	87	32°C	7	25	22	28.7	25.3
			10	15	7	17.2	8.0
			13	23	20	26.4	23.0

*Dissemination rate **(c)** for legs or head in that order is provided as [(**b** divided by **a**) × 100%] where numerator **b** corresponds to number of infected legs or head respectively. (n): number of infected abdomens in each day post infection.*

Regardless of the EIT, the MIR of *Ae. aegypti* sampled from Coastal Region was significantly ($p<0.05$) higher than those sampled from Western Kenya, with no statistical differences observed for the coastal *Ae. aegypti* at EIT 26 and at 32 °C. Meanwhile, the MIR of *Ae. aegypti* from the Western Kenya Region (26°C) was significantly ($p< 0.05$) affected by the EIT, with mosquito reared at EIT 32°C exhibiting higher MIR than those reared at EIT 26°C. The 2-way interactions between region, EIT and DPI significantly affected the MIR ($p<0.03$). Also, a significant ($p<0.0002$) interactive effects of the region, EIT and DPI on MIR was established. The disseminated infection rates for the CHIKV in *Ae. aegypti* in the legs (DIR-L) was higher in the coastal mosquitoes regardless of the EIT while those from western Kenya dissemination rates was significantly ($p<0.0037$) higher at higher EIT of 32°C. There was no significant difference ($p>0.05$) between region and EIT on the dissemination of CHIKV from the midgut to the head, indicating that the trends in the infection rates by CHIKV remained similar at the coastal and western region regardless of the EIT. Notably, there was no significant ($p>0.05$) difference between region, EIT and DPI on DIR on the legs and head.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Arboviruses are ecologically complex viruses, and interactions between larval mosquitoes and their aquatic environment can influence adult transmission dynamics. Moreover, due to the impact of climate on vector ecology, competence and their risk of transmitting viruses may be sensitive to projected changes in global temperatures. In this study, the effect of ambient temperatures and changes of EIT on the risk of vector transmission and competence of two *Ae. aegypti* populations for CHIKV was evaluated. The results provide evidence that the incubation temperatures of vector directly impact virus transmission by influencing the likelihood of infection and dissemination of CHIKV. The MIR of *Ae. aegypti* sampled from the coastal area with ambient temperature of 32°C was higher than those sampled from the western Kenya region that has ambient temperature of 26°C regardless of the EIT. Meanwhile, for *Ae. aegypti* mosquitoes from lower ambient temperature of 26°C, there was increased MIR when EIT was increased from 26°C to 32°C. Climatic effects, particularly elevated temperature, on virus development in vector mosquitoes also could have enhanced transmission efficiency (Watts *et al.,* 1987). Lack or limited rainfall in this area leads to residents storing water in their households (especially in areas with water storage) and since CHIKV thrives in areas that lack water, it creates a conducive breeding ground. Hence, the proximity of container breeding mosquitoes to man could be a major factor that triggers disease occurrence.

Although the original source of CHIKV in the Lamu outbreak was not determined, widespread domestic water storage at the time could have facilitated vector breeding and human contact. Infrequent water replenishment has been shown to increase domestic *Ae. aegypti* populations in coastal Kenya (Subra, 1983). Therefore, this effect of low temperatures could support the study undertaken suggesting that virus transmission is

likely to be affected more by higher environmental temperature (Purse *et al.,* 2005). It has earlier been noted that temperature may affect virus transmission in areas where the vectors are present. For instance, an increase in environmental temperature for adult mosquitoes reduces the EIP most likely due to an increase in the metabolism of the adult mosquito and replication speed of the virus (Davis, 1932; Chamberlain & Sudia, 1955; Purse *et al.,* 2005). Equally, temperature changes experienced in the immature stages of the mosquito development before infection may affect vector virus interactions by changing physical and physiological characteristics of mid-gut barriers which would impact virus infection and transmission (Hardy *et al.,* 1990; Westbrook *et al.,* 2010). Previous studies which have established that ambient temperature affects the biological processes of mosquitoes and plays a key role in modulating mosquito vector competence for pathogens (Lambrechts *et al.,* 2011; Murdock *et al.,* 2012; Lefevre *et al.,* 2013). Previous studies have indicated that increases in adult-holding incubation temperatures have usually been associated with enhanced vector competence (Kay *et al.,* 1989; Richards *et al.,* 2009; Anderson *et al.,* 2010). However, some studies have identified reduced vector competence and activity in nature associated with increases in incubation temperature (Kramer *et al.,* 1983; Muturi *et al.,* 2011).

It has long been recognized that increases in incubation temperature reduce the extrinsic incubation period (the time from initial acquisition of pathogens until transmission is possible) (Chamberlain & Sudia, 1955), which render virus transmission more likely under such incubation period. Along the same lines, increases in temperature reduce the adult lifespan of mosquitoes and may impinge transmission (Kay *et al.,* 1989; Hardy *et al.,* 1980; Turell, 1993). Temperature effects may drastically alter risk of disease transmission, especially under conditions where the extrinsic incubation period approaches the lifespan of the mosquito. This result differs with other systems where arboviral vector competence was reduced in female mosquitoes that were reared at higher compared to lower temperatures (Kay *et al.,* 1989; Hardy *et al.,* 1980). However, vector capacity of a mosquito population is a complex phenomenon that is influenced by a number of other factors such as host seeking behavior and longevity of the infected

mosquitoes apart from temperature and inherent factors (Lutomiah *et al.,* 2013) and thus further studies are recommended on how these factors can combine to affect the transmission dynamics of chikungunya in particular and arboviruses in general. The 2004- 2005 outbreak of Chikungunya in Kenya documented at least 1,300 cases. This outbreak reached the Comoros at the end of 2004 before spreading to the Indian Ocean islands in 2005. The affected areas were Lamu and Mombasa in the Coast Region. A seroprevalence study conducted in October 2004 suggested 75% of the population in Lamu had been affected (Sergon *et al.,* 2007; Sergon *et al.,* 2008).

During the recent outbreak in May 2016 in Mandera East sub-county, it was observed that the CHIK isolates were grouped with isolates from the post 2005 Indian Ocean islands, Asia and Europe. Mogadishu having experienced a chikungunya outbreak in areas bordering Kenya could explain cross border infection due to human movement and geographical proximity between the two regions. Mosquito susceptibility to arbovirus infection resides primarily in the midgut and can vary greatly between mosquito species and geographical strains of the same species and even within individuals of the same population (Hardy *et al.,* 1983). Vector competence, which is the capacity of an arthropod to acquire an infection and transmit it to a subsequent host, can greatly vary among individuals and between populations (Turell *et al.,* 1992) and has been previously linked to genetics (Mercado-Curiel *et al.,* 2008) as well as climate variables such as temperature (Turell, 1993; Dohm *et al.,* 2002).

Disseminated infection is generally accepted as a measure of a mosquito's ability to transmit a virus through biting (Turell *et al.,* 1992; Turell, 1993). The rate of dissemination, when expressed as a percentage of the number of mosquitoes infected, may provide information about the effect of a "midgut escape barrier" moderating whether gut infections are able to disseminate into the hemolymph. In the current study, the dissemination rates of infected *Ae. aegypti* for CHIKV in the legs was high at higher ambient temperature regardless of the EIT. Notably, the disseminated infection rates for the CHKV in *Ae. aegypti* in the legs was higher in mosquitoes from the coastal region

regardless of the EIT while those from western Kenya had dissemination rates significantly higher at higher EIT. These results suggest that the midgut barriers preventing dissemination are strongly influenced by the ambient and rearing temperature. Thus, it can be speculated that there is an increased midgut escape barrier in mosquitoes derived from the higher rearing temperatures. At temperature of 26°C during the adult stage resulted in the lowest rates of viral dissemination. Rates of dissemination were higher at higher EIT relative to lower holding temperatures of adults. These results corroborate observations found for laboratory studies examining susceptibility to dengue virus infection and length of the extrinsic incubation period in *Ae. albopictus* and *Ae. aegypti* (Watts *et al.,* 1986; Rohani *et al.,* 2009; Xiao *et al.,* 2014). However, there was no association between vector dissemination in between the midgut and the head. The explanation for these observed effects of mosquitoes with disseminated infections is not entirely clear, but it does suggest complex effects of temperature on virus infection and dissemination and by extension, mosquito competence.

5.2 Conclusion

The findings show that both mosquito populations are susceptible to CHIKV. The findings suggest an inefficient transmission ability of CHIKV by Trans Nzoia *Ae. aegypti* population but supports disseminated infection by Lamu population. This could explain why there has been no active CHIKV transmission in Trans nzoia. As evidenced from the results temperature has a significant effect on the vector competence and these variations may help explain the distribution and spread of CHIKV. Increase in temperatures due to climate change could impact the spread of the virus through local vectors into susceptible host populations becomes inevitable. Although these results show differences in vector competence, other factors (mosquito densities, feeding behavior, mosquito survival rates) composing the vector capacity, are needed to assess more accurately the risk of CHIKV transmission alongside virus titers in the context of dissemination.

5.3 Recommendations

Since no mosquito and none of the inoculated ones were fed on naïve chicken to show transmission it is not possible to tell either of these *Ae. aegypti* from both Lamu and Trans Nzoia regions was actually able to transmit CHIKV by bite. Hence, additional studies are needed to know whether these mosquitoes from these two different regions are involved in the transmission cycle of CHIKV in Kenya. Future studies should also explore the connection between larval rearing temperature-infection patterns observed in the laboratory and patterns in the field, and how climate and climate change related factors may continue to impact the mosquito larval environment and the epidemiology of CHIKV.

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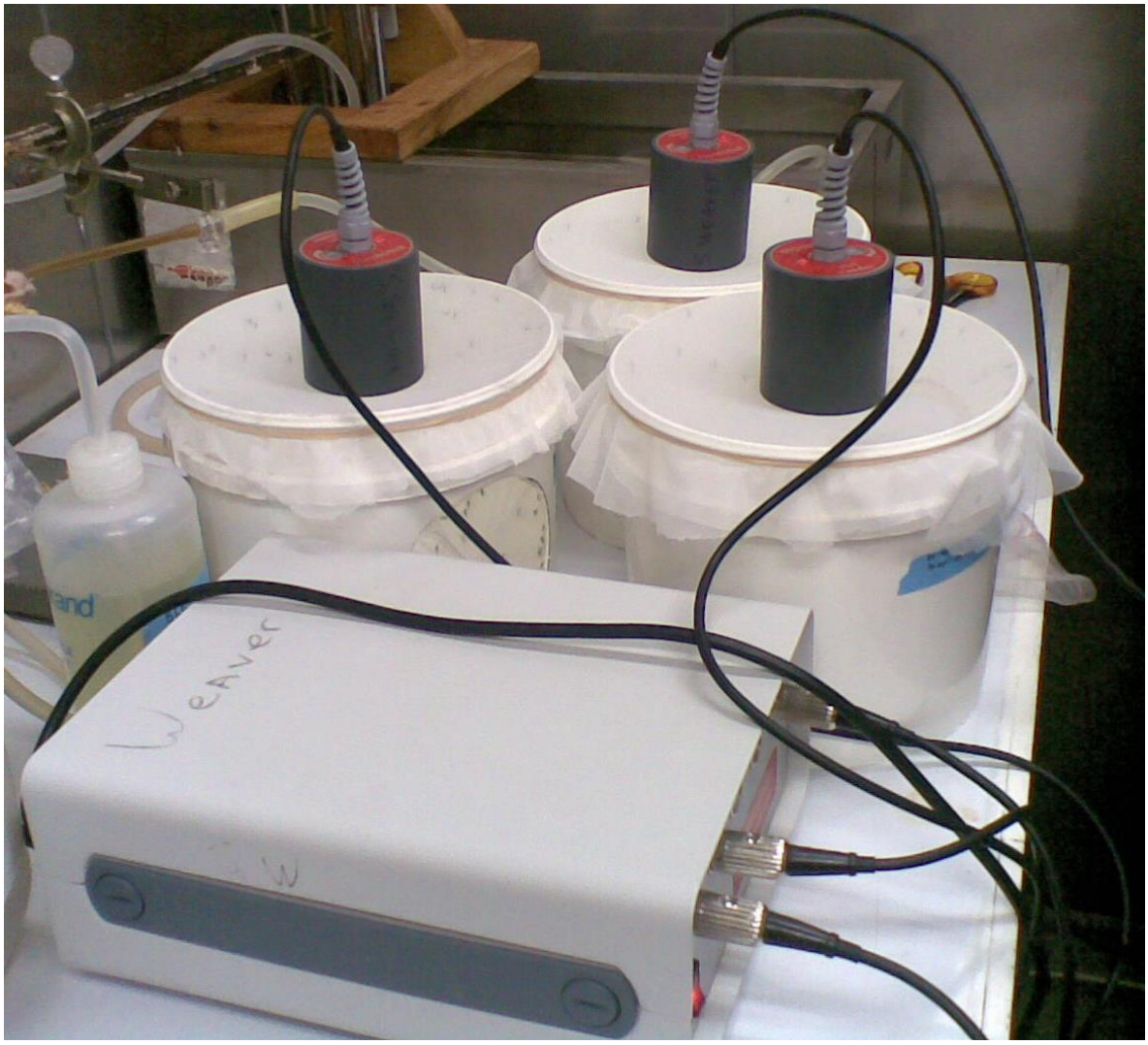
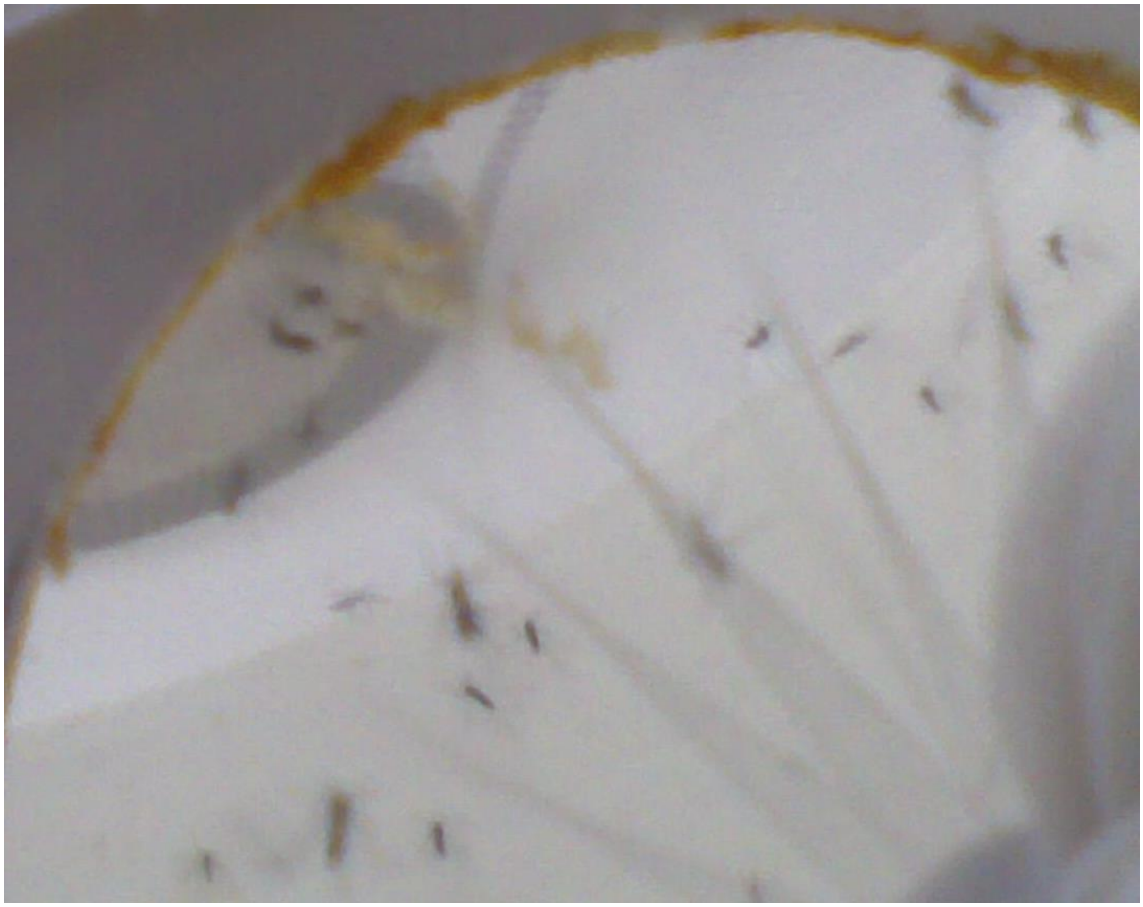
APPENDICES

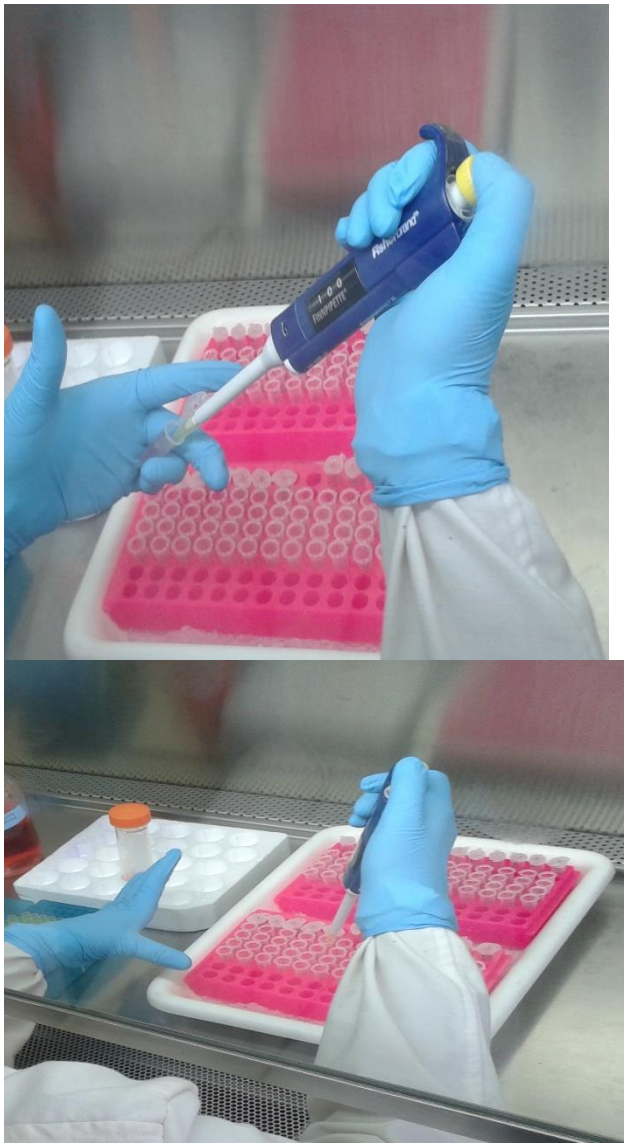
Appendix I: Aedes aegypti mosquitoes feeding through a hemotek membrane feeder



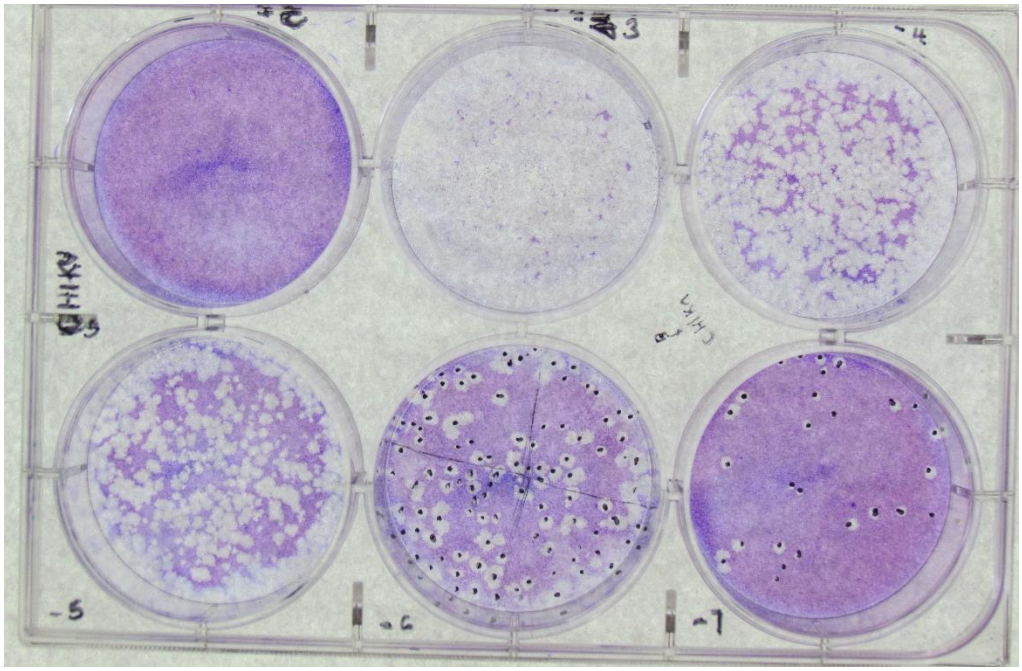
Appendix II: Engorged Aedes *aegypti* mosquitoes after 45 minutes feeding



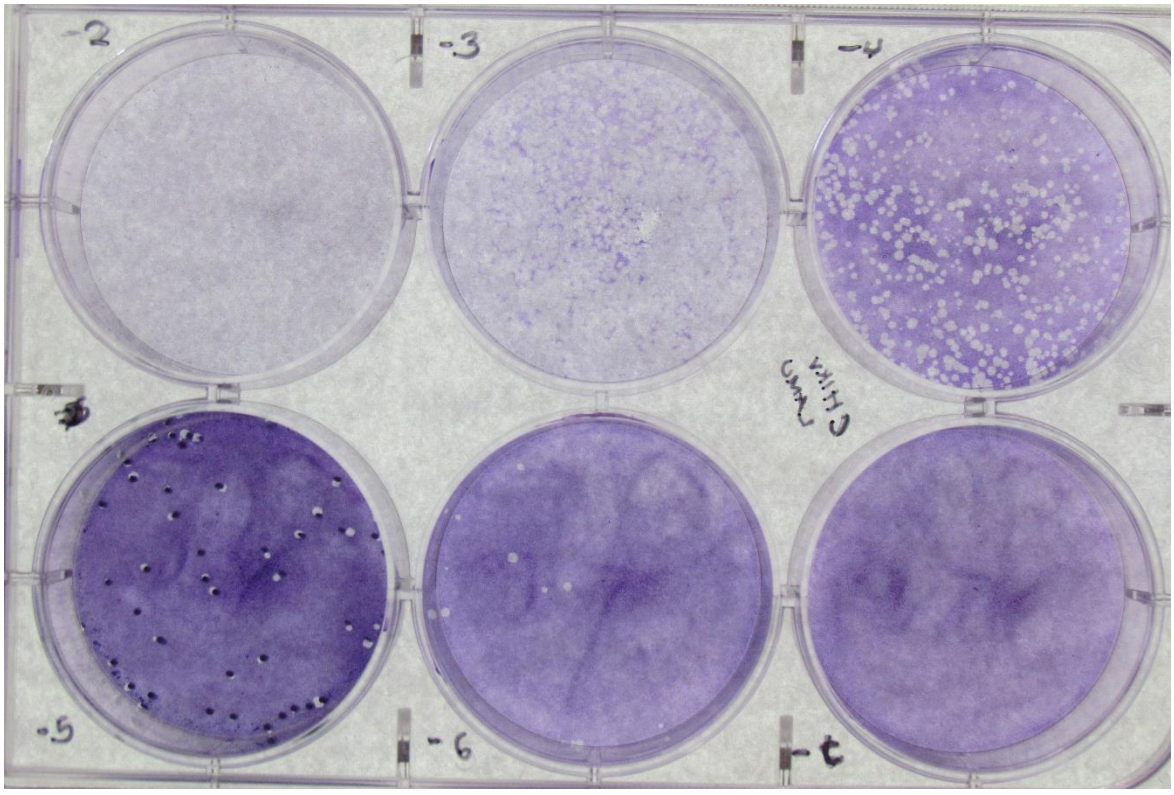
Appendix III: Drawing of the homogenate (sample) and performing serial dilutions



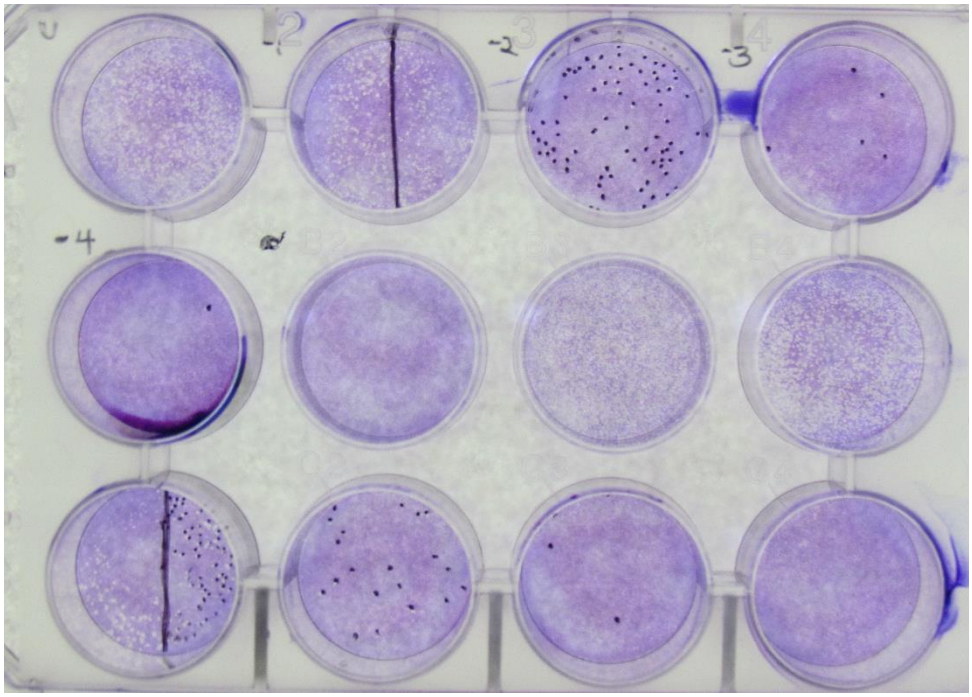
Appendix IV: 6 well- plate plaque assay of Chikungunya virus



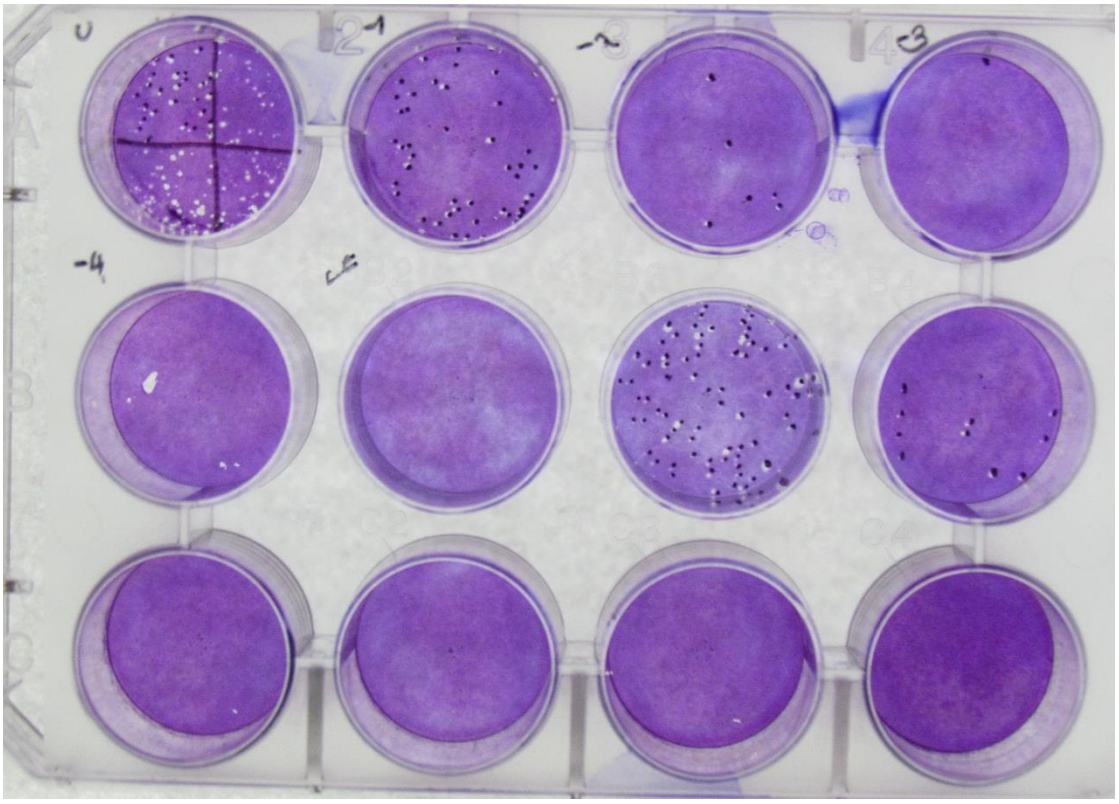
Appendix V: 6 well- plate plaque assay of blood+ Chikungunya virus



Appendix VI: 12 well- plate plaque assay of mosquito of abdomen homogenate (Western Kenya Aedes aegypti)



Appendix VII: 12 well- plate plaque assay of mosquito of abdomen homogenate (Lamu Aedes aegypti)



Appendix VIII: Clearance letter from KEMRI Scientific Steering Committee (SSC)

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

ESACIPAC/SSC/101091 8th November, 2012

Sophiah Mbaika

Thro'
Director, CVR
NAIROBI

Forwarded

REF: SSC No. 2345 (3rd Revised) – Evaluation of Coastal and Western Kenya populations of *Aedes aegypti* as Chikungunya virus vectors under varying environmental conditions

Thank you for your letter received on 5th November, 2012 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval

FOR: Sammy Njenga, PhD
SECRETARY, SSC

Encl(s)

CENTRE FOR VIRUS RESEARCH 12 NOV 2012 P.O. Box 54628 - NAIROBI

Appendix IX: Clearance letter from KEMRI Animal Care and Use Committee (ACUC)

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O. Box 54628 - 00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 02 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2726115
Email: cvr@kemri.org

KEMRI/ACUC/ 01.08.12 **3rd August 2012,**

Sophiah Mbaika

Thro,
The Director
Centre for Virus Research
Nairobi.

Sophiah Mbaika,

RE: Approval for SSC 2345 "Evaluation of Coastal and Western Kenya populations of *Aedes aegypti* as Chikungunya virus vectors under varying environmental conditions " Protocol

Following the resubmission of the above mentioned proposal to the ACUC addressing the issues raised earlier the committee recommends that you proceed with your work after obtaining all the other necessary approvals.

The committee expects you to adhere to all the animal handling procedures in KEMRI as described in your proposal.

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

c.c

Appendix X: Clearance letter from KEMRI Ethics Review Committee (ERC)

KENYA MEDICAL RESEARCH INSTITUTE

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Tel: (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **February 27th, 2013**

TO: **SOPHIA MBAIKA**

THROUGH: **DR. FRED OKOTH**
DIRECTOR, CVR,
NAIROBI

DIRECTOR
CENTRE FOR VIRUS RESEARCH
21 P. O. Box 54628
NAIROBI

DEAR MADAM,

RE: **SSC PROTOCOL NO. 2345: (REV): EVALUATION OF COASTAL AND WESTERN KENYA POPULATIONS OF AEDES AEGYPTI AS CHIKUNGUNYA VIRUS VECTORS UNDER VARYING ENVIRONMENTAL CONDITIONS**

Reference is made to your letter dated 21st February, 2013.

This is to inform you that the Ethics Review Committee (ERC) finds that the issues raised at the 211th meeting of the KEMRI/ERC meeting held on 6th February 2012 have been adequately addressed. Consequently, the study is granted approval for implementation effective this **26th February 2013**

Please note that authorization to conduct this study will automatically expire on **February 23, 2014**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **December 16th, 2013.**

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the SSC and ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study.

Sincerely,

RAB

DR. ELIZABETH BUKUSI,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

20 MAR 2013
P.O. Box 54628 - NAIROBI