VECTOR COMPETENCE OF AEDES SIMPSONI BROMELIAE AND AEDES VITTATUS POPULATIONS FROM KILIFI AND WEST POKOT, KENYA FOR CHIKUNGUNYA VIRUS

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Vector Competence of Aedes Simpsoni Bromeliae and Aedes Vittatus Populations from Kilifi and West Pokot, Kenya for Chikungunya Virus

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2021

DECLARATION

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

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DEDICATION

This project work is dedicated to my lovely Daddy, Daniel Musili and my late Mum, Josephine, my dear wife Elizabeth, my lovely daughter, Jasmine.

To Daddy: Thank you for your love, care, endurance and prayers

To Elizabeth, Jasmine: You're the source of my inspiration.

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ABBREVIATION AND ACRONYMS

°C	Degree Centigrade
ACUC	Animal Care and Use Committee
ATCC	American Type Culture Collection
BSC2	Biological Safety Cabinet 2
BSL2	Biosafety Level 2
CHIKV	Chikungunya virus
CO_2	Carbon dioxide
CPE	Cytopathic effect
DEET	N, N- Diethyl- meta- toluamide
dNTPs	Deoxynucleotide triphosphates
EIP	Extrinsic incubation period
EIT	Extrinsic incubation time
ERC	Ethical Research Committee
F_1	First filial generation
FBS	Fetal bovine serum
HM	Homogeinization media
KEMRI	Kenya Medical Research Institute

L: D	Light: Day
MEB	Midgut escape barrier
MEM	Minimum Essential Media Eagles
MIB	Midgut infection barrier
$NaHCO_3$	Sodium bicarbonate
PBS	Phosphate buffer solution
PFU	Plaques forming unit
PPE	Personal Protective Equipment
RH	Relative Humidity
RNA	Ribonucleic acid
SERU	Scientific and Ethics Review Unit
SGI	Salivary gland infection
VHF	Viral Haemorrhagic fever
YF	Yellow fever
YFV	Yellow fever virus
WHO	World Health Organizatio

ABSTRACT

Kenya has experienced outbreaks of chikungunya in the recent past with the most recent occurring in Mandera in the northern region in May 2016 and in Mombasa in the coastal region from November 2017 to Febuary 2018. Despite the reported outbreaks in Kenya, vector competence studies have only been conducted on *Aedes aegypti* mosquitoes*;* however the role played by other mosquito species in transmission and maintenance of the chikungunya virus in endemic areas remains unclear. This study sought to determine vector competence of rural *Ae. bromeliae* and *Ae. vittatus* mosquitoes in the transmission of chikungunya virus, focusing on Kilifi and West Pokot regions of Kenya.This is a laboratory based experimental study that involves oral infection of mosquitoes with infectious blood meal and observing the outcome of the infection. Four day old female mosquitoes were fed orally on chikungunya virus-infected blood at a dilution ration of 1:1($10^{6.4}$ plaque-forming units [PFU]/ml) using artificial membrane feeder (hemotek system) for 45 minutes. The engorged mosquitoes were picked and incubated at 29-30°C ambient temperature and 70-80% relative humidity in the insectary. At 5, 7 and 10 days post infection the mosquitoes were selected and carefully dissected to separate the legs and wings from the body and their proboscises individually inserted in the capillary tube containing minimum essential media (MEM) to collect salivary expectorate. The resulting homogenates and the salivary expectorates were tested by plaque assay to determine virus infection, dissemination and transmission potential of the mosquitoes. A total of 515 female mosquitoes (311 *Ae. bromeliae* and 204 *Ae. vittatus*) were exposed to the East, central and Southern Africa (ECSA) lineage of chikungunya virus. *Ae. vittatus* showed high susceptibility to the virus ranging from 75-90% and moderate dissemination and transmission rates ranging from 35-50%. *Ae. bromeliae* had moderate susceptibility ranging from 26-40% and moderate dissemination and transmission rates ranging from 27-55%. Findings from this study showed that strains of *Ae. vittatus and Ae. bromeliae* populations from West Pokot Kenya and Kilifi county respectively were competent vectors for CHIKV. Overall, about 70% of the *Ae. vittatus* and about 40% of the *Ae. bromeliae* that ingested >10 6.4 plaque-forming units of virus/mL became infected and about 30% of the virus-exposed mosquitoes transmitted virus to a capillary tube. Vector competence remains a prerequisite in risk assessment, surveillance and control of vector.. This study shows that both *Ae. vittatus* and *Ae. bromeliae* populations from West Pokot and Kilifi counties of Kenya are competent vectors of chikungunya virus. Based on these results, the two areas are at risk of virus transmission and outbreaks in the event of virus introduction. Therefore, this study underscores the need to institute vector competence studies for different populations of potential vector species across the country as a means of evaluating risk of transmission of the emerging and re-emerging arboviruses in diverse regions of Kenya.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Chikungunya virus (CHIKV) is a vector-borne virus of genus *Alphavirus* and family *Togaviridae*, which is principally transmitted from human to human by *Ae. aegypti* and *Ae. albopictus*. The first CHIKV outbreak was documented in Makonde village in Tanzania in 1956 (Lumsden, 1955; Robinson, 1955) and since then, various outbreaks have been experienced in more than 60 countries in Africa, Asia, Europe and America (Staples and Fischer, 2014; Zeller *et al* 2016a). In Africa high infection was reported in union of Comoros island during 2004- 2005 outbreak (Powers and Logue, 2007), Congo during 1998-2000 outbreak (Njenga *et al*., 2008) and Mauritius and Madagascar in 2005 and 2006 respectively (Ravi *et al.,* 2006). CHIKV is re-emerging in Kenya, after the 2004-2005 outbreaks in Lamu Island(Sergon *et al*., 2008) since then several outbreaks have been reported in the country, the northeastern and coastal Kenya experienced CHIKV outbreak in 2016 and late 2017 to early 2018 respectively (Konongoi *et al.,* 2018; WHO, 2018a).

Chikungunya virus strains are classified into three distinct genotypes: Asian, West African, and East/Central/ South African (ECSA). This virus causes chikungunya fever, an acute febrile illness characterized by severe arthralgia, fever, skin rash, and arthritis-like pain in small peripheral joints that lasts for weeks or months, joint swelling and conjunctivitis (Beltrame et al.,2007; Chhabra*et al.,* 2008; Vega-Rúa *et al*., 2014). *Ae. aegypti* and *Ae. albopictus* have been implicated in the CHIKV transmission cycle in the African region and other parts of the world based on vector competence studies (Agha *et al.,* 2017a) and viruses isolated from infected field collected mosquitoes from various areas during outbreaks *(Bonilauri et al. 2008; Leo et al.*,2008; Sang *et al.*, 2008). International travels and global expansion of the two main CHIKV urban mosquito vectors, *Ae. aegypti* and *Ae. albopictus*, have enhanced the ability of the virus to spread

to new regions where environmental conditions are permissive for viral transmission (Lanciotti *et al.,* 2007; Zammarchi *et al.,* 2016; Zeller *et al.*,2016a). The extrinsic incubation period (EIP) in mosquitoes infected with CHIKV ranges from 2 to 9 days, with an average of 3 days in the tropics such as East Africa (Rudolph *et al.*,2014). High prevalence has been reported during CHIKV outbreaks (Konongoi *et al*., 2018; Sergon *et al.*, 2008). In addition, previous studies have reported high seroprevalence rates of 59% of CHIKV infection in Busia county and 24% in Malindi in Kilifi county, Kenya (Mease *et al*., 2011).

Ae. simpsoni consists of a complex of mosquito species including vectors of important arbovirus diseases like yellow fever. In Kenya, *Ae. bromeliae* is the dominant species of the *Ae. simpsoni* complex found in the peridomestic areas. Study involving the ecology and vector competence of *Ae. vittatus* and *Ae. bromeliae* on chikungunya have been conducted in Senegal (Diagne et al., 2014). In Rabai, Ae. *bromeliae* breeds in the domestic and peridomestic areas while *Ae. lilii* breeds in the forest (Agha*et al*., 2017b; Walter *et al*.,2014). *Ae. bromeliae* population from coast has been proven and confirmed to be a competent vectors of yellow fever in Kenya (Ellis *et al*., 2012). This *species* preferably feeds on human hosts for their blood meal source, maintaining the virus in the rural cycle (Ellis *et al*., 2012) and mostly breed in not only on water reservoirs held by plants, including trees holes and plant leaf axils (Mukwaya *et al*., 2000), and in artificial water containers within the homestead thus increasing the risk of virus transmission (Bown and Bang, 1980; Trpis*et al*., 1971).

Ae. vittatus is a tropic and subtropics species that is abundant mostly in rocky areas, presence in African forest galleries both in arid and sub arid areas, but can also be found in villages near forests (Diallo *et al*., 2012a). *Ae. vittatus* female mosquitoes have both diurnal and nocturnal activity with a significant crepuscular peak at 12.00 pm to 10.00 pm (Diagne *et al*., 2014; Diallo *et al*., 2012b). The species is both anthropophilic and zoophilic and has shown to bite a wide range of vertebrate hosts including primates and small rodents, based on locations the species tend to have a strong anthropophilic tendency (Lee and Moore, 1972). Aedes vittatus breeds mostly on natural habitat such as rock

pools/holes and tree holes during the rainy season, but in absence of these breeding sites the vector breed in domestic areas especially in household water-holding containers (Diagne *et al*., 2014). The vector has shown to have high susceptibility to infection, dissemination and transmission of the West Africa lineage of CHIKV in the conducted laboratory experiments (Diagne *et al*., 2014; Diallo *et al*., 2012b). *Ae. vittatus* and *Ae. bromeliae* have potential to expand their distribution and abundance due to their ability to adopt to the human dwelling using available artificial breeding habitats since they have shown to breed in domestic containers in absence of their preferred breeding sites (Agha *et al.*, 2017a; Agha *et al*., 2017b; Diallo *et al*., 2012a).

Determination of vector competence of mosquito population is a key parameter in evaluating the risk of CHIKV transmission and spread in diverse ecological areas of Kenya. Despite several outbreaks of CHIKV in Kenya, focus is usually on *Ae. aegypti* in the past and no vector competence studies have been conducted to determine the role played by other mosquito species in transmission and maintenance of the virus. I evaluated the competence of *Ae. bromeliae* population from Rabai sub-county in Kilifi County and *Ae. vittatus* populations from Kacheliba sub-county in West Pokot County of Kenya, as an important factor in assessing the risk of transmission of ECSA lineage of CHIKV in rural area of the Kenyan regions. This was important to provide the necessary baseline data to inform the National and County public health sectors on best vector control interventions to prevent an outbreak in case of increased risk of virus transmission.

1.2 Statement of the Problem

CHIKV is a global re-emerging mosquito-borne *alphavirus* transmitted by *Ae. aegypti* mosquitoes in Africa and *Ae. albopictus* in Asia causing major epidemics that causes significant morbidity and mortality in humans. The first ever chikungunya-related deaths having been documented on Reunion Island in 2004/2005 outbreak that affected a large population along the Indian ocean, there is no vaccine or treatment of the disease hence the vector control and supportive therapy is the only effective method to control the disease. Sporadic outbreak of CHIKV have been reported in the country for instance, in

2004/2005, an outbreak of chikungunya was reported in Lamu, coastal Kenya, 2016 in North Eastern and 2018 in Mombasa, The virus has remained a major public health problem especially in coastal region and Mandera county. The mosquito implicated was *Ae. aegypti* that is typically responsible for human CHIKV transmission although other vectors such as *Ae. bromeliae* and *Ae. vittatus* were also collected during the outbreaks. There is no information about the competence of *Ae. bromeliae* and *Ae. vittatus* in Kenya to transmit CHIKV hence the need to evaluate the vector competence of the two species. Kilifi county neighbours Mombasa county that experiences sporadic outbreak of chikungunya hence the need to determine the efficiency of *Ae. bromeliae* which is dominant in Rabai, in west pokot sero prevalence of chikungunya was high for the primate and human sample surveyed during the arbovirus suirveillance hence the need to determine the vector competence of *Ae. vittatus* which is the most dominant species in the region.

1.3 Justification

International travels and the global expansion of the two main urban mosquito vectors of CHIKV, *Ae. aegypti* and *Ae. albopictus*, have enhanced the ability of the virus to spread to new regions where environmental conditions are permissive. The disease epidemics are highly linked to the urban vector, *Aedes aegypti* but the role played by other rural vectors such as *Ae. Vittatus and Ae. bromeliae* remains unclear in our country which are now widely distributed in Kenya. This mosquito species are rapidly expanding its range and it's currently abundant in and around rural areas in Kenya. High population density, increased global travels, lack of adequate resources for vector control and hygiene adds to the vulnerability of humans to CHIKV infection. The risk of CHIKV re-emerging in Kenya is high due to its geographical proximity to endemic areas, such as Reunion Island, Comoros and Asian countries, increased domestic and international travel and there is no vaccine for the virus hence increasing the chance of new infections. The virus evolves rapidly and the knowledge on the vector competence for the other mosquito's populations for CHIKV is scanty. In the previous epidemics, CHIKV has demonstrated high ability to spread and infect large populations hence needs to understand the vectorial capacity of

other mosquitoes in endemic areas to transmit CHIKV. No study has been done to determine ability of *Ae. bromeliae* and *Ae. vittatus* mosquito to transmit CHIKV, this is the first important step in assessing the risk of CHIKV transmission and outbreaks in Kenya. The results will guide in developing appropriate targeted monitoring and control strategies.

1.4 Research Question

1. What are the preferred breeding areas of *Ae. bromeliae* populations in Kilifi County and *Ae. vittatus* Populations in West Pokot county in Kenya?
2. What are the infection and dissemination rates of CHIKV in experimentally infected *Ae. bromeliae* and *Ae.vittatus* populations from Kilifi and West Pokot county respectivelyin Kenya?
3. What are the transmission rates of CHIKV in experimentally infected *Ae. bromeliae* and *Ae. vittatus* populations from Kilifi and West Pokot county respectively in Kenya?

1.5 Null Hypothesis

Aedes bromeliae and Ae. vittatus mosquito populations from Kilifi and West pokot respectively are not competent vectors for chikungunya virus

1.6 Objectives

1.6.1 General Objective

To determine the vector competence of *Ae. bromeliae* and *Ae. vittatus* mosquito populations from Kilifi and West pokot respectively for chikungunya.

1.6.2 Specific Objectives

1. To determine the breeding preference of *Ae. bromeliae* in Kilifi County and *Ae.vittatus* populations from West Pokot County in Kenya

2. To determine the susceptibility to Infection and dissemination of *Ae. bromeliae* and *Ae. vittatus* populations from Kilifi and West pokot respectively for chikungunya virus chikungunya virus
3. To determine the transmission of CHIKV by experimentally infected *Ae. bromelia* and *Ae.vittatus* populations from Kilifi and West pokot respectively for chikungunya virus.

CHAPTER TWO

LITERATURE REVIEW

2.1 Chikungunya virus

Chikungunya virus is an RNA *alphavirus* belonging to the family *Togaviridae* transmitted primarily by *Aedes* mosquitoes. Chikungunya infection causes an acute febrile illness, chikungunya fever characterized by severe arthralgia, fever, skin rash, and arthritis-like pain in small peripheral joints that lasts for weeks or months, this results to high morbidity (Beltrame *et al.*, 2008; Chhabra *et al*., 2008). The virus is endemic in Africa and Asia with imported cases reported in other continents such as Europe and America (Chhabra *et al*., 2008). The genome analysis shows CHIKV form three major lineages which represent adaptation to regional conditions, an East, Central, South African (ECSA) lineage, a West African (WA) lineage, and an Asian lineage (Weaver *et al*., 2014). International travels, commerce, tourism and local travels have resulted in the introduction of viruses, particularly members of the ECSA and Asian lineages, into new geo-graphic areas, resulting in epidemic outbreaks (Weaver & Forrester, 2015)

2.2 Chikungunya virus Genome

Chikungunya virus contains a single-stranded, positive-sense RNA genome approximately 11.5 kb in length that encodes four nonstructural proteins and three main structural proteins: the capsid and two envelope glycoproteins, E1 and E2, which form spikes on the virion surface. E2 binds to unknown cellular receptors to initiate cell entry through endocytosis, and E1 includes a fusion peptide, exposed at low pH in endosomes, which initiates the release of nucleocapsids into the host-cell cytoplasm (Khan *et al*., 2002). Chikungunya virus genome encodes 9 genes, consisting of coding sequence for non-structural polyproteins (precursor for nsP1-nsP4 proteins), structural polyproteins (precursor for C, E1-3 and 6K proteins), and polyadenylation site, flanked by 5’ and 3’ sequences (Khan *et al*., 2002). In previous outbreaks prior to the 2006 epidemic in south

east Asia and India, the 3 genotypes were restricted to the geographical areas denoted by their names (Kalantri *et al*., 2006a). The Asian genotypes have shown to have high degree of nucleic acid sequence diversity while the African strain exhibit low sequence diversity, and have been shown to undergo genetic micro-evolutions even during the event of an epidemic (Schuffenecker *et al*., 2006a). The East African genotype has been isolated from the 2005 epidemic in the Indian Ocean Islands and the strains currently circulating in India which has been attributed by international travels (Yergolkar *et al*., 2006a, Yergolkar *et al*., 2006b). The structure of chikungunya virus-like particles in complex with strongly neutralizing antibody Fab fragments (8B10 and 5F10), the Fab fragments of antibody 8B10 extend radially from the viral surface and block receptor binding on the E2 glycoprotein. In contrast, Fab fragments of antibody 5F10 bind the tip of the E2 B domain and lie tangentially on the viral surface. Fab 5F10 fixes the B domain rigidly to the surface of the virus, blocking exposure of the fusion loop on glycoprotein E1 and therefore preventing the virus from becoming fusogenic(Porta *et al*., 2016).

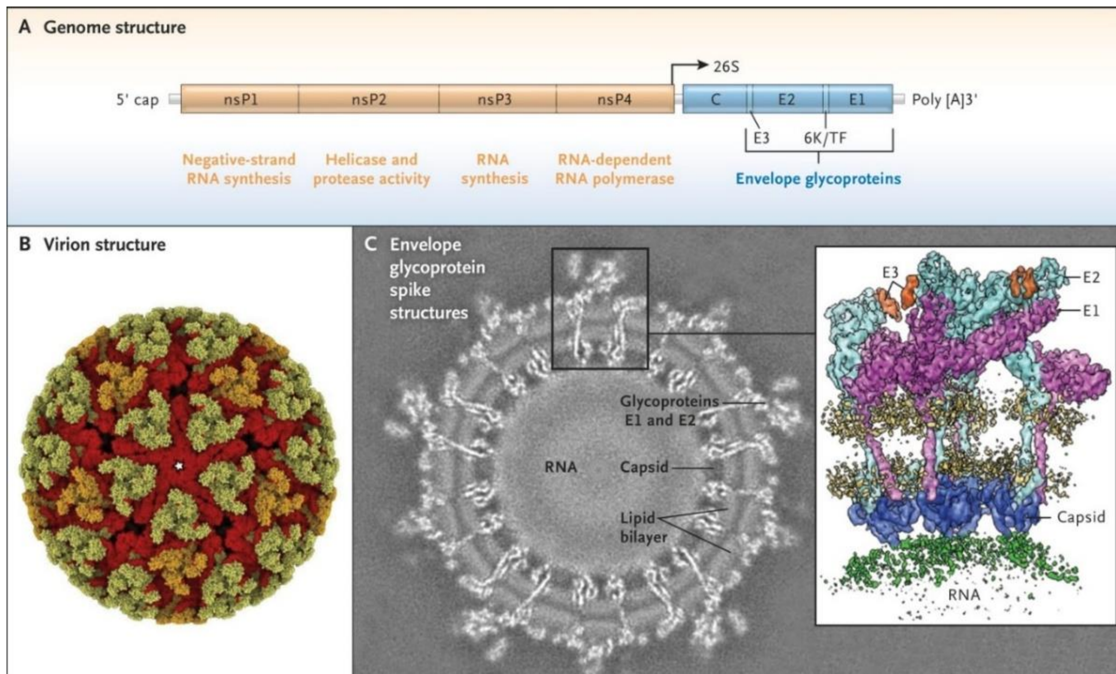


Figure 2.1: Chikungunya Virus Genetic and Physical Structure. Courtesy of Scott C. Weaver, 2015

Panel A shows the organization of the chikungunya virus genome, including its nonstructural proteins 1 through 4(nsP1–nsP4) and structural proteins C (capsid), E1–E3 (envelope glycoproteins), and 6K/TF (6K and TF [transframe] are alternative translation products of the same gene).

Panel B shows the structure of the virion (image courtesy of Felix Rey, Institut Pasteur, Centre National de la Recherche Scientifique). Panel C shows spike-protein predicted structures based on atomic resolution structures of the envelope glycoproteins2 and high-resolution cryoelectron microscopic reconstructions of chikungunya virus and other alphavirus particles.

2.3 Origin and spread of CHIKV

The chikungunya virus was first isolated in Tanzania during dengue like illness in the year 1952-1953 (Robinson, 1956; Ross, 1956). The first emergence of the virus into the urban cycle during the modern scientific era occurred between 1879 and 1956, when a member of the eastern, central, and southern African (ECSA) enzootic lineage was introduced into Asia (Renault *et al*., 2012), In 2004, an outbreak involving another ECSA lineage progenitor began in coastal Kenya before spreading to several Indian Ocean islands and to India, where it caused explosive epidemics involving millions of people (Renault *et al*., 2007). Subsequently, infected air travelers arrived in Europe, Asia, and the Americas, and local transmission ensued in Italy, metropolitan France, and many countries in South and Southeast Asia (Sergon *et al*., 2008; Zeller *et al*., 2016b). Currently available data on chikungunya virus strains and their sequences do not clarify whether this introduction into Asia occurred in the 19th century or more recently. This epidemic strain, called the Asian lineage, caused outbreaks in India and Southeast Asia and continues to circulate in the latter region (Sergon *et al*., 2008). Chikungunya virus circulates in forested regions of sub-Saharan Africa in an enzootic transmission cycles involving nonhuman primate hosts and forest mosquito vector, urban transmission cycle involving human and vectors, and also can occur through intermediate cycle which occurs as a result of spillover of enzootic transmission cycles (Gargan *et al*., 1988). The unprecedented magnitude of the outbreaks was believed to probably influenced by several factors including increased air travel, which permitted rapid spread (Angelini *et al*., 2007). Increased urbanization in most of the tropics and subtropics countries with denser human and urban mosquito populations for instance the invasion of *Ae. albopictus* from its native Asia into islands in the Indian Ocean basin, Africa, and southern Europe, which was facilitated by increased global commerce and a series of adaptive mutations in the new Indian Ocean lineage (IOL) chikungunya virus strains, this has shown to enhance virus transmission by *Ae. albopictus* which was not implicated as a major vector in previous Asian epidemics (Diallo *et al*.,1999).

2.4 CHIKV epidemics

The first ever chikungunya outbreak was reported in Makonde, Tanzania in 1952 where the name chikungunya came from due to the characteristic symptoms associated with the virus (Robinson, 1956; Ross, 1956). The CHIKV caused outbreaks in various countries in eastern, southern and west Africa(Weaver & Forrester, 2015). The largest scale of chikungunya outbreak was reported in Kenya 2004/2005 (Chretien *et al*., 2007) which spread to the Indian ocean islands, where more than 250,000 cases were reported in La Reunion in 2005-2006 (Njenga *et al*., 2008; Sang *et al*., 2008; Sergon *et al*., 2008; Weaver, 2014). The outbreak strain from the western Indian Ocean Islands was related to previous East, Central, and South-African isolates (Schuffenecker *et al*., 2006b). CHIKV infection has progressively spread to different countries in the Indian Ocean region and Asian countries along the coastal line (Sergon *et al*., 2008). Since then imported cases of CHIKV infection in travelers returning from affected areas were reported in several European and American countries (Beltrame *et al*., 2007; Lanciotti *et al*., 2007). Infrequent water replenishment (expected because of drought) increases domestic *Ae. aegypti* populations and widespread domestic water storage could also have facilitated vector breeding and human contact (Chretien *et al*., 2007) in coastal Kenya. The most recent outbreak of chikungunya in Kenya occurred in Mandera in north eastern and in Mombasa in coastal region in 2016 and 2018 respectively (WHO, 2018b)

2.5 Epidemiology of CHIKV

2.5.1 Transmission of CHIKV and risk factors

Chikungunya virus is mainly transmitted through bites of infected *Aedes* mosquitoes mainly *Ae. aegypti* and *Ae. albopictus*; this species 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. Mobility of people influenced by the globalization of trade and ease of travel is largely responsible for the wide dissemination of the virus. The epidemic in the Indian Ocean islands of Comoros,

Mauritius, Seychelles and Reunion island lends credence to this suggestion. These islands, popular among tourists, attract about 1.5 million people every year (Volpe *et al*., 2006). After a major epidemic broke out in these islands in 2005, several tourists returning from the islands to Italy, Southern France and Spain were viraemic (Volpe *et al*., 2006). *Aedes aegypti* is also known to multiply by laying its eggs in a puddle of water in discarded tires. Such tires, when transported across countries, can spread the infected *Aedes* species and their eggs worldwide (Bonn, 2006).

2.5.2 Clinical Manifestations of CHIKV infection

Chikungunya virus causes human disease characterized by acute fever, headaches, rash, myalgia, arthralgia and febrile illness with joint pain (P. Jupp, 1988). Illness is rarely fatal but can be severe and prolonged, and lead to other complications (Marimoutou *et al*., 2012). The incubation period has shown to range from two to ten days in adults, the disease preferably affects adults and children whose immunity is compromised (Yergolkar *et al*., 2006b). The fever and skin rash has shown to be short-lasting but the joint pains may recur or linger for a long time sometimes for as long as 3 years after the onset of disease (Pastorino *et al*., 2004). Drug reaction often causes Papular or maculopapular rash on the arms and the abdomen after 48 hours of illness (Kalantri *et al*., 2006b).

2.5.3 Treatment and vaccination of CHIKV infection

There is neither established antiviral treatment nor licensed vaccine for CHIKV infection (Couderc *et al*., 2009). Treatment is purely symptomatic and is based on non-steroidal anti-inflammatory drugs (Briolant *et al*., 2004). Synergistic efficacy was reported between interferon-α and ribavirin on CHIKV in vitro. A trial in southern Africa failed to confirm the efficacy of chloroquine on arthralgia (Delogu and de Lamballerie, 2011). The development of vaccines that can induce lasting protective immunity has been on going over the years. However many trials have been stopped due to limited demand and funding (Kam *et al*., 2012). A live CHIKV vaccine was developed via 18 serial passages through MRC-5 cell cultures by the U.S Army (Levitt *et al*., 1986) but due to the inherent

instability (potential reversion) of RNA genomes coupled with little attenuating mutations, further work was halted.curently there are several chikungunya vaccines candidates that are in late stage of development some in phase 2 and 3 clinical trialsfor instance the National institute of health (NIH) supported vaccine candidates.

2.5.4 Prevention of transmission of CHIKV

Vector control programs that involve environmental management are highly effective in reducing the transmission 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 vectors (Heintze *et al*., 2007). The resultant decline in the vector-host cycle can reduce transmission of the virus with a consequent decline in the incidence of the disease. Evidence suggests that when communities actively take part in abolishing the breeding places of vectors (destruction, alteration, disposal or recycling of domestic containers); the density of larvae is significantly reduced. Personal protection such as applying insect repellant to the exposed skin can keep out *Ae. aegypti,* a daytime biter. Thirty percent DEET in insect repellants have shown to provide an average of 5 hours of complete protection against vector bites after single application on the exposed skin (Fradin and Day, 2002).

2.6 Vectors of Chikungunya virus

Chikungunya virus is transmitted by mosquitoes of the genus *Aedes* such as *Ae. aegypti* and *Ae. albopictus* in Asia (Turell *et al*., 1992) and *Ae. frucifer*, *Ae. luetocephalus*, *Ae. taylori* in Africa (Diallo *et al*., 1999). This virus has a sylvatic cycle in Africa between wild primates and forest *Aedes* spp. mosquitoes and urban cycle, while in Asia and the Indian Ocean it cycle exclusively between humans and *Aedes spp*. mosquitoes (P. Jupp, 1988).

The vectors of CHIKV in the Indian Ocean basin are *Ae. aegypti* and *Ae. albopictus* (Townson *et al*., 2008). *Ae. aegypti* and *Ae. albopictus* are implicated in the CHIKV

transmission cycle due to vector competence (Dubrulle et al., 2009) and infected field isolates (Leo *et al*., 2009 Sang *et al*., 2008), 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 from Africa, India and other countries in southeast Asia (Leo *et al*., 2009). However, *Ae. albopictus*, the Asian tiger mosquito, has also played a role in the transmission of CHIKV during the recent outbreaks in India, the reunion island in the Indian Ocean, and Italy (Mavale *et al*., 2010).

2.6.1 *Ae. bromeliae* and *Ae. vittatus*

Ae. simpsoni complex mosquitoes are vectors of yellow fever virus in Kenya and are known to be associated with cultivated microhabitats (Ellis *et al*., 2012) and preferentially breed in phytotelma, i.e., water reservoirs held by plants, including tree holes and plant leaf axils (Haddow, 1945, 1948), although they are also found in artificial water containers in absence of their preferred breeding areas (Bang *et al*., 1980; Trpis *et al*., 1971). Gillet proposed that *Ae. simpsoni* were tree hole breeders in the ancestral state; the large-scale cultivation of bananas, faro, and pineapples may have provided new breeding sites with continually available water sources, resulting in spatial reproductive isolation owing to oviposition site. *Ae. bromeliae* is the dominant species in Kenya where it preferably breeds in natural habitats in peridomestic areas, *Ae. lilii* species breeds mostly on forest and known to transmit yellow fever (Huang, 1979). In coastal region, *Ae. bromeliae* and *Ae. lilii* are separated by the microhabitats where the *Ae. bromeliae* breeds in the domestic area and the *Ae. lilii* breeds in the forest (Walter *et al*., 2014). *Ae. bromeliae* is a competent vector of yellow fever in Kenya and it prefers feeding on humans where it maintains the virus through the urban lifecycle (Ellis *et al*., 2012).Further reproductive isolation may result from the timing of ovipoisition. Breeding of the sylvan ecotype is closely correlated to rainfall and oviposition rates markedly decline during the dry season, when phytotelma are unavailable for the mosquitoes to oviposit (Bang *et al*., 1980). The availability of oviposition sites for the domestic ecotype during the dry season does not pose the same seasonal constraints on breeding of the domestic clade, potentially enhancing the level of

reproductive isolation between sylvan and domestic forms (Diagne *et al*., 2014) *Ae. vittatus* breeds mostly in the rock pools/holes and is a vector of yellow fever virus where it is involved in sylvatic cycle of the virus (Diagne *et al*., 2014).The taxonomic status of *Ae. vittatus* has been discussed several times. Firstly, it was included in the genus *Stegomyia* due to morphological similarities with *Ae. aegypti*, and then it was included in subgenus Aedimorphus (Huang, 2001; Huang & Rueda, 2015). Recently, the species was transferred to the new subgenus Fredwardsius, based on a distinct combination of characters that distinguish it from other subgeneric taxa (Reinert, 2000). Generic status was proposed for Fredwardsius in a recent phylogenetic study (Reinert *et al*., 2004), but for the time being we prefer to retain it as a subgenus of *Aedemorphus*. *Ae.(Aedimorphus) vittatus* is the only species of the subgenus *Aedimorphus* involved in yellow fever virus (YFV) transmission in Africa, the *Aedes vittatus* has been found associated with YFV in nature and its ability to transmit was proven experimentally (Ngoagouni *et al*., 2012). Nevertheless, it is still considered to be an accessory vector, since very little information has been obtained on its involvement during YF epidemics. It was only incriminated in the epidemic that occurred in Sudan(Onyango *et al*., 2004) in 1940 and was suspected to have played an accessory role during outbreaks that occurred in the Jos Plateau, Nigeria, in 1959 and 1969 (Lee & Moore, 1972). The species has pronounced seasonal dynamics, with an early peak of density at the beginning of the rainy season, which suggests a limited role in sylvatic transmission because the virus tends to emerge towards the end of the rainy season(Diez-Fernandez *et al*., 2018). *Ae. vittatus* is a savannah species that is abundant in rocky areas. It is especially prevalent in West African forest galleries, but it is also common in villages near forest. Females have daily and nocturnal activity with a significant crepuscular peak. They bite a wide range of vertebrate hosts, with a strong anthropophagic trend in specific locations. *Ae. vittatus* breeds mostly on natural habitant mainly the rock pools/holes and tree holes during the rainy season while in absence of this breeding area the vector have shown to breed in domestic area especially in household containers holding water for domestic use (Diagne *et al*., 2014). Vector competence of the West Africa lineage of CHIKV has shown the vector to have high susceptibility to infection, dissemination and most importantly transmission of the virus, *Ae. vittatus*

mosquitoes have been shown to have infection rate of between 50-100% (Diagne *et al*., 2014).

2.7 Vector Competence

Vector competence is the inherent capacity of an arthropod to acquire and maintain an infection and transmit it to a subsequent host, which can greatly vary among individuals and between populations (Turell *et al*., 1992). Vector competency is determined by isolation of an arbovirus from a naturally infected vector, laboratory demonstration of vector infection following feeding on viremic blood, laboratory evidence of viral transmission during blood feeding and evidence of contact between vector and vertebrate host in nature. One of the principal determinants of the distribution of vector-borne diseases is climate (Rogers & Randolph, 2006), Vector competency is further affected by extrinsic factors like rainfall, vertebrate host population, humidity and temperature. Temperature and humidity affect vector development and longevity. In addition, these two factors influence the rate of virus multiplication in the vector and the time necessary for completion of viral incubation that ultimately results in the ability of the vector to transmit by bite. In addition, these two factors influence the rate of virus multiplication in the vector and the time necessary for completion of viral incubation that ultimately results in the ability of the vector to transmit by bite. High virus doses have also been determined to increase vector competence by overcoming barriers to infection for different vector species and viruses(Mahmood *et al.,*2006). Other factors include vector age and strain for example, young *Aedes aegypti* were found to have a higher survival probability compared to older mosquitoes hence more likely to complete the extrinsic incubation period necessary for virus transmission. Duration of the extrinsic incubation period is important in the epidemiology of arboviruses since it determines the length of time a vector must survive after infection for it to efficiently transmit the virus. Thus higher vector population densities or prolonged survival times may be required to maintain transmission cycles of vectors with long extrinsic incubation periods and vice versa (Hardy *et al*., 1983). In mosquito vectors, development, survival and arbovirus replication are greatly influenced by temperature (Delatte *et al*., 2009; Dohm*et al*., 2002; Parham *et al*., 2015). Ambient

temperature can affect the arbovirus dynamics within the mosquito vector by altering the length of the extrinsic incubation period (EIP), which is the time between ingestion of an infectious blood meal and when transmission to a subsequent host is possible (Patz *et al*., 1996). 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 the replication speed of the virus. Temperature may also limit viral transmission in areas where the vector is present, but the temperature precludes efficient transmission (Westbrook *et al.,* 2010). Species of mosquitoes from different geographic regions differ in biological trait and morphologic characteristics. *Culex pipiens* has been implicated in RVF outbreak in Egypt (Hoogstraal *et al.*, 1979), *Aedes vexan* in west Africa (Traore-Lamizana *et al*., 2001; Zeller *et al*., 1997) and Saudi Arabia (P. Jupp *et al*., 2002), *Aedes caspius* in Egypt as an enzootic vector (Gad *et al*., 1999). A competent arboviral vector must be susceptible to infection, abundant, long lived and able to blood feed on and become infected by both amplification and dead-end host. Short-lived mosquitoes rarely serve as competent vectors because they do not have sufficient time to complete the extrinsic incubation period. Laboratory assessment of vector competence can provide an insight on the potentiality of field collected mosquitoes as disease vectors. Such competency studies have been performed for RVF virus (Moutailler *et al*., 2008; Turell *et al*., 2007). Laboratory experiments have shown that several mosquito species can be orally infected with viruses and can subsequently transmit the infection through blood feeding (Turell *et al*., 1996). These studies, some which use mosquito species not implicated in transmission cycles, have shown the potential for local transmission in the event that the virus is introduced into the environment (Turell & Kay, 1998). Given that mosquito control methods differ for different species, it is of public health importance to identify which species of mosquitoes are competent vectors that may be involved in the natural transmission cycle so that appropriate control measures can be applied.

The transmission potential of a vector borne disease by vectors have been used to predict risks of outbreaks, evaluate vector control strategies, and to compare strains of a pathogen,

parameters such as extrinsic incubation period and survival rate act as a means for evaluating infection rates in the event of an outbreak (Anderson and Rico-Hesse, 2006).

2.8 Transmission barriers

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 (Smith *et al*., 2005). Mosquito have shown to salivate during blood feeding because of saliva that contains different substances counteracting the host hemostatic response that prevent blood coagulation and enhance vasodilatation (Schneider and Higgs, 2008). Arboviruses infect the mosquito midgut following ingestion of a viremic blood, replicate, disseminate to the salivary glands, and emerge into saliva to be transmissible to a vertebrate host when the mosquito bites. The midgut and salivary glands act as barriers to virus infection and escape(Hardy *et al*., 1983). The Salivary anti-haemostatic components of mosquitoes may differ from one species to another , the salivary armoury and probing behavior have shown to efficiently counteracts their preferred hosts(Calvo *et al*., 2009a, Calvo *et al*., 2009b). It has also been reported that saliva is able to enhance viral infections, Mosquito saliva promotes extensive cutaneous edema that leads to retention of viruses at the feeding site this hijacks immune cells and facilitates viral spread from the bite site, Mosquito saliva disrupts endothelial barrier function to enable blood feeding, which facilitates virus dissemination and cell migration suppressing the innate immune response of human host cells which subverts host adaptive immune responses (Fong *et al*., 2018a; Schneider & James, 2006) the saliva contains a collection of chemicals which include anticoagulants to prevent blood clotting, vasodilators to keep blood vessels wide, and anesthetics to prevent humans from sensing the mosquito, Saliva also contains components that enhance viral replication, dissemination, and pathogenesis by inducing an inflammatory response that inadvertently promotes infection by providing new cell targets for infection(Fong *et al*., 2018b).

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. Once ingested by its mosquito host, a virus must overcome several obstacles if it is to establish itself and 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) to infect 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 (Beaty & Thompson, 1978). The time required for an arbovirus and viruses to infect, multiply, and escape from the mosquito mesenteron into the hemocoel has shown to vary depending upon the climate, mosquitoes generation, type and dose of virus, mosquito species, and temperature of extrinsic incubation (EIT)(Chepkorir *et al*., 2014; Mbaika *et al*., 2016). The ingested virus must multiply in the mesenteron before infection of the salivary glands can occur (Turell *et al*., 2010). 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, thus, it's becoming increasingly evident that the vector competence of mosquitoes for arboviruses is quite complex and is associated with multiple barrier systems (Turell *et al*., 2010). Previous studies have documented the existence of a midgut (mesenteron) infection barrier associated with refractoriness when some arboviruses are ingested by certain mosquito species . Studies have shown the existence of other barriers to infection apart from the midgut barrier. Hardy et al proposed that a series of barriers prevent or reduce further dissemination of virus at various times between its appearance in the lumen of the midgut and its eventual shedding into the salivary glands (Hardy *et al*., 1983). The mosquito lumen is separated from the hemocoel by a single epithelial layer of cells surrounded by a porous multilayered membrane, the basal lamina. The first virus proliferation occurs in the epithelial cells if a sufficient viral dose is ingested. However, infection fails to establish or may be established only at very high viral dose if a mosquito is refractory to a given arbovirus. The second barrier is posed by the basal lamina and is

termed the "mesenteronal escape" barrier. A third barrier is the salivary gland infection barrier. Both were experimentally demonstrated with Western equine encephalitis virus in *Culex tarsalis* (Kramer *et al*., 1981). In 20-- 30% of infected *C. tarsalis,* the virus did not multiply to normal levels in the mesenteron and did not disseminate into the hemolymph regardless of the length of time. In another 20-45% of infected mosquitoes, the virus multiplied normally and disseminated into the hemolymph, but failed to infect the salivary glands. However, the salivary gland infection barrier in some of these mosquitoes was overcome after an extended incubation period showing that this barrier is both time and dose dependent. Individuals of some species are unable to transmit the virus during blood feeding even at optimal conditions for highly competent vector species (Chamberlain & Sudia, 1961; Jupp *et al*., 1981). This might be related in part to the infecting dose of virus. Studies have shown that a reduction in the infective dose of virus reduces arboviral transmission (P. G. Jupp, 1974).

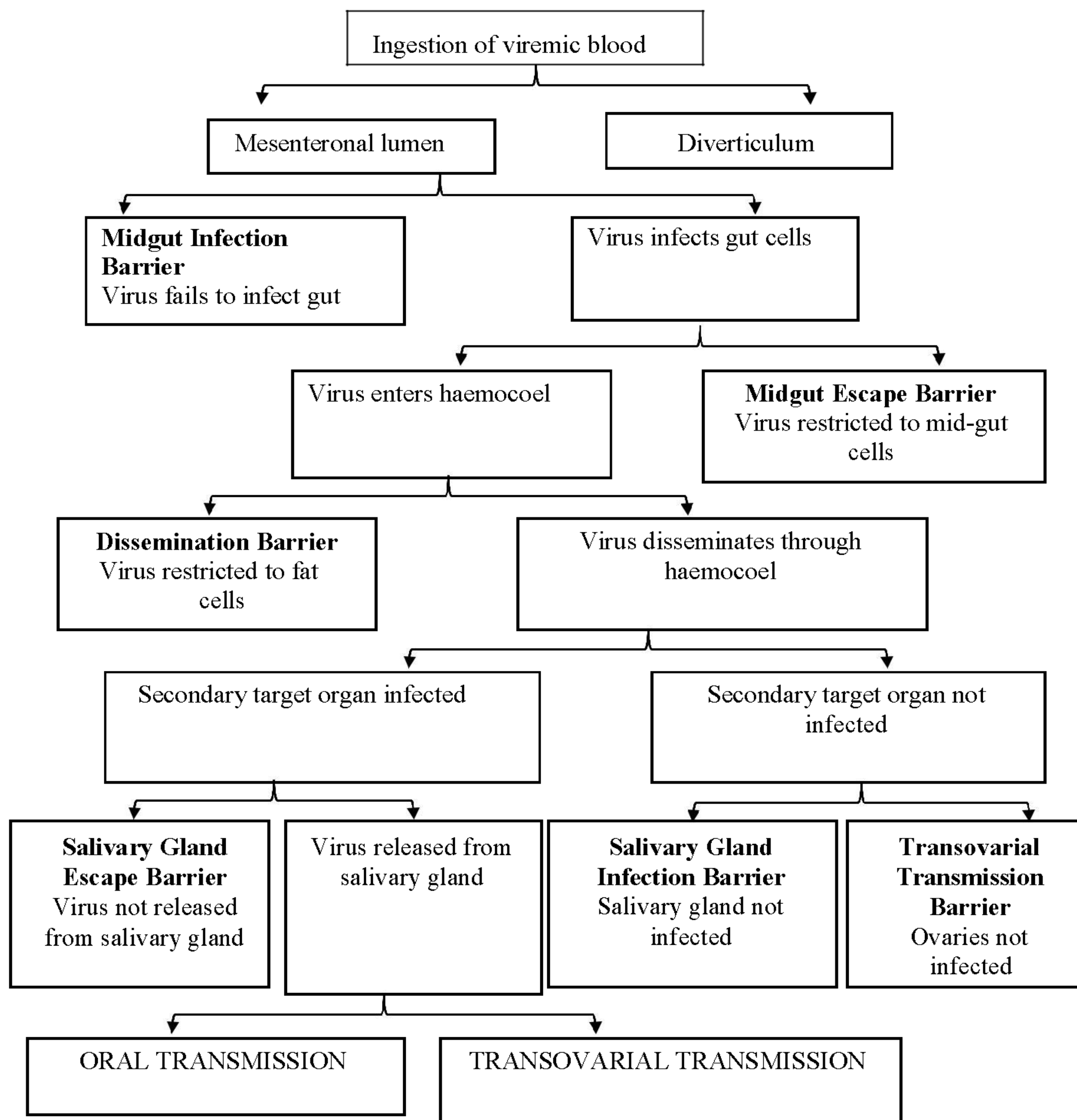


Figure 2.2: Hypothesizes transmission barriers (bold) to arbovirus infection (Adapted from Hardy et al. 1983).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

The study was conducted in Rabai sub County of Kilifi County in the coastal region of Kenya and Kacheliba Sub County in West Pokot County (Figure 1). Kilifi County lies at latitude of 3.63°S and a longitude of 39.85°E, it receives mean annual rainfall of approximately 88.25 mm and 82% relative humidity and temperature of 30°C per year (Chepkorir *et al.,* 2014). Four site were sampled for the immature *Ae. bromeliae* this includes Mbarakani, Bengo, Changombe and Kibarani (Figure1). The County receives bimodal pattern of rainfall, long rains occurs between April and July, with the highest rainfall occurring in the month of May, short rains are experienced on the month of November and December. Minimum temperatures are always above 20°C, the maximum temperatures ranges between 30°C to 34°C.

West Pokot County lie at latitudes 24^0 40'North, and 1^0 7'North and longitudes 34^0 37'East and 35^0 49'East in the Rift Valley in Kenya, it borders Uganda to the West and the following counties, Trans-Nzoia to the South, ElgeyoMarakwet and Baringo to the South East, and Turkana to the North and North East. It covers an area of 9,169.39 Sq. The county experiences bimodal rainfall, long rain season are experienced on the month of May and June with a mean daily temperature of 32°C, rainfall of approximately 60.25 mm and 82% relative humidity, due to climate change the rainfall patterns have become difficult to predict.

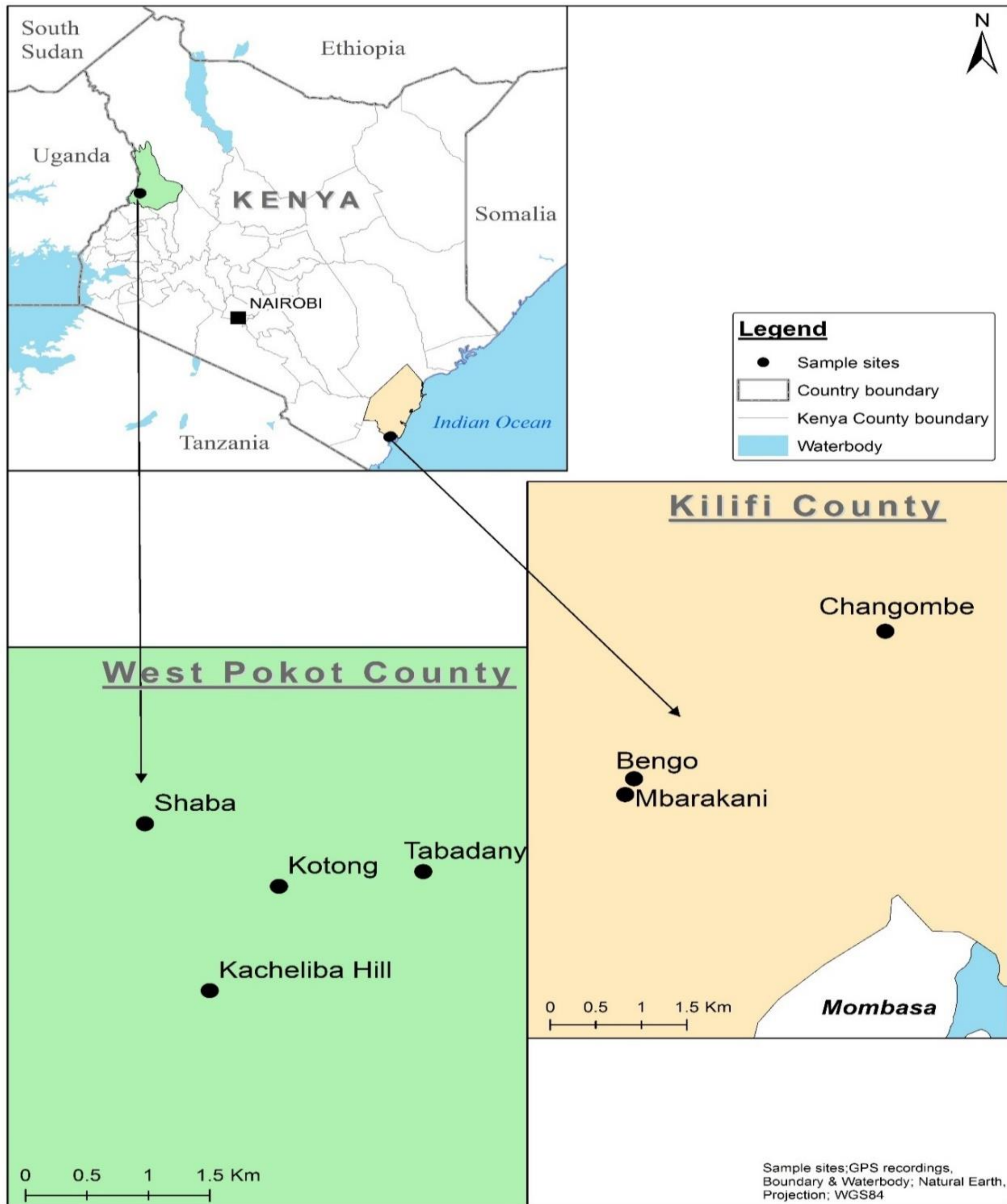


Figure 3.1: Map of Kenya showing the study site where the vectors were collected in west Pokot and Kilifi county

3.2 Study Design

This was laboratory- based experimental study that involes oral infection of mosquitoes collected from selected regions in Kenya with CHIKV and observing the oucome of the experiment.

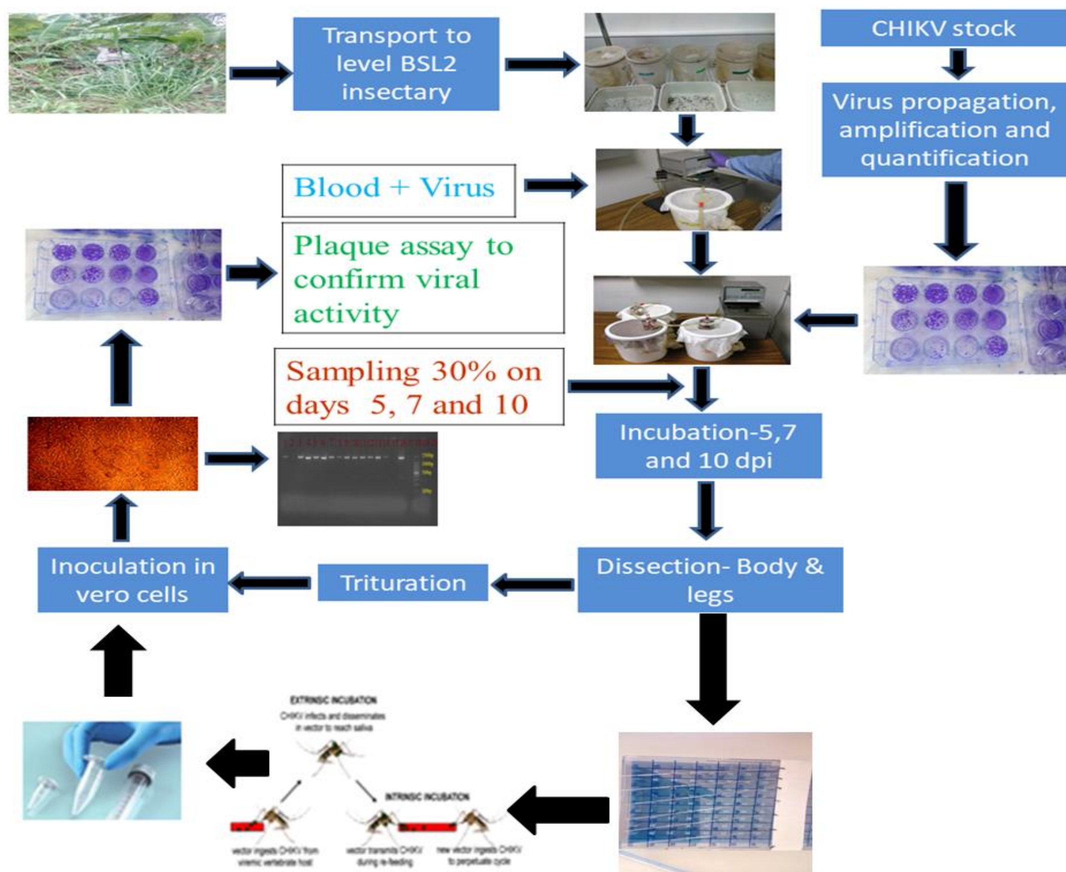


Figure 3.2: Schematic representation of the experimental design.

3.3 Mosquito Populations

Aedes bromeliae eggs, larvae and pupae were collected from peridomestic areas in the four villages in Rabai sub-county namely Mbarakani, Bengo, Changombe and Kibarini in Kilifi County, the eggs were collected using ovitraps that consisted of black ovicups lined with oviposition paper and half-filled with water. *Aedes vittatus* larvae and pupae were

collected from rock pools/holes and tree holes in peridomestic and forest areas of Kacheliba sub-county namely Kacheliba hills, Shaba, Kotong and Tabadany in West Pokot County. Field collected eggs were briefly dried on a damp cotton wool to induce diapause, and transported to Level 2 (BSL2) insectary at Kenya medical research institute (KEMRI) for colonization.

3.4 Mosquito collection

The mosquitoe were collected from the selected household in the study area, the selection for the household was purposive sampling where i looked at household with peridomestic and forest area with natural habitants. After the home owner/residence gave the permission to do sampling in their private land, the ovitraps were placed in the peridomestic areas for four days to allow the mosquitoes to lay eggs. Larvae and pupae were collected from both natural habitats mainly rock pools/holes and tree holes, plant axils especially bananas and flower axils as well as artificial containers using larval sampling tools. The immauture mosquitoes were collected once from each household and each habitant type recorded on the sampling form and coordinates for each household taken using the Germin Geographical positiong system. The sampling was done once in each household sected for the study.

Figure 3.3: Immature mosquitotes collection and sampling from water held on Banana leaf axils(A), tree hole (B) and rock pools (C) and sisal leaf axils (D) in Kilifi and West Pokot County

3.5 Mosquito rearing and identification

To avoid selection of a single female oviposition, several larval collections from the same area were mixed in the laboratory, all collected larvae and pupae were reared to adults in the field laboratory and then transported to the KEMRI insectary for identification. Oviposition papers with eggs were collected and briefly dried on moist cotton wool to induce diapauses before transportation to Biosafety Level 2 (BSL2) insectary at Kenya medical research institute (KEMRI). In the insectary, the oviposition papers with eggs

were dispensed in water to allow hatching and the emerging larvae were fed on fish fingerlet meal (Tetramin baby) until pupation. The pupae were transferred in small cups containing water to 4 litre plastic cages with netting material for eventual development to adults. The adults were knocked down at -20°C for 45 seconds and morphologically identified using identification keys (Edwards, 1941; Huang, 2001; Jupp & McIntosh, 1990; Jupp, 1996) under a dissecting microscope to select *Ae. bromeliae* and *Ae. vittatus* for use in the study. The adult mosquitoes were provided with 10% glucose solution on cotton wool and maintained at temperature between 28-32ºC, 70-80% relative humidity and 12:12 hour light: dark (L: D) photoperiod. In order to stimulate egg production the mosquitoes were fed on clean laboratory mice placed on top of the cage for 45 minutes. The eggs collected were hatched into F_1 (First filial generation) and adult mosquitoes were maintained as described above.

Plate 3.1: Immature in the larval rearing tray and adult mosquitoes in the cages breeding in the level 2 Insectary

3.6 Virus and virus amplification

3.6.1 Cell lines

Vero cells are cell line extracted from the kidney of the African green monkey and is used primarily in virus replication studies and plaque assays. The Vero cells that were used to culture all the virus strains were from ATCC (ATTC® Number: CCL-81™) and had been passaged ten times. They were cultured in T-25 cm2 culture flask overnight using Eagle's Minimum Essential Media (MEM) (Sigma-Aldrich, St. Louis, MO) with Earle's salts and reduced NaHCO3, supplemented with 10% heat-inactivated fetal bovine serum (FBS), (Sigma-Aldrich), 2% L-Glutamine (Sigma-Aldrich), and 2% antibiotic/ antimycotic solution with 10,000 units penicillin, 10mg streptomycin and 25µg amphotericin B per ml (Sigma-Aldrich) incubated at 37^0C in a humidified incubator with 5.0 % CO2, to form confluent monolayers.

3.6.2 Virus isolate

The Lamu001 strain of an ECSA lineage of CHIKV which was isolated from human during the 2004–2005 outbreak in Lamu island (Njenga *et al.*, 2008), which was archived in the VHF laboratory was used for the study. The cell cultures which had been grown in 25cm2 tissue culture flasks were washed with sterile PBS pre-warmed in the water bath at 37^0C. Two hundred microlitre of thawed sample was inoculated in the monolayer followed by incubation in 5% CO_2 incubator at 37^0C for 1hr with frequent rocking at an interval of 15 minutes in order to allow for virus adsorption. After incubation, the infected cells were maintained in 5ml Minimum Essential Medium (MEM), (Sigma-Aldrich, St. Louis, MO) with Earle's salts and reduced $NaHCO_3$, supplemented with 2% heat inactivated foetal bovine serum . The cells were monitored twice a day for cytopathic effects (CPE). After two days 80% cytopathic effect (CPE) was observed after, The infected cells were then harvested by spinning down and the supernatant used for molecular assay while the remainder was kept as virus stocks in the -80°C freezer in the VHF laboratory (Reiskind *et al*., 2008).

3.6.3. Harvesting of the virus

When about 80% CPE was observed, the virus was harvested by freezing down the infected cells in the -80 ℃ freezer overnight to lyse the cells thus releasing the viral particles. Then the frozen cells were thawed on ice and the suspension was transferred into a 15 ml centrifuge tube and centrifuged (KUBOTA KS 5000 centrifuge) at 277xg for 5min to sediment the cells. The supernatant containing the virus was placed in 1ml cryovials tubes in 0.5ml aliquots and stored at -80℃ freezerThe cell cultures were harvested and tested by reverse transcriptase polymerase chain reaction (RT-PCR) after isolating RNA from the cell culture supernatants.

3.6.4 RNA Extraction

The QIAamp Viral RNA Minikit (QIAGEN, Hilden Germany) was used to extract viral RNA in culture suspension that was harvested after 24 hours post inoculation according to the manufacture's protocol with 40 µl of RNA being obtained for subsequent use.

3.6.5 RT-PCR Assays

In a 200 µl PCR tube, 10 µl of the extracted sample RNA was mixed with 2 µl of 50 ng/µl random hexamer primer and 1 µl of 10 mM deoxynucleotide solution (dNTPs), and incubated in a thermocycler for 5 minutes at 65˚C and immediately chilled for 1 minute at 4˚C. The following components were then added to the PCR tube: 4 µl of 5X First Strand Buffer (Invitrogen), 1 µl of 0.1MDTT, 1 µl of RNAse OUT(40 U/µl) and 1 µl of Superscript III Reverse transcriptase (200 U/µl). The mixture was then incubated in a thermocycler for 5 minutes at 25˚C, 50 minutes at 50˚C and 15 minutes at 70˚C. A total of 20 µl cDNA was obtained. A two-step reverse transcription PCR (RT-PCR) was carried out. First strand cDNA was synthesized by combining 5ng of random Hexamer primer (Invitrogen) and 10µl of RNA and the mixture was incubated at 70°C for 10 minutes to denature the RNA and also to allow the primer to anneal to the RNA. The mixture was cooled down at 4°C for 5 minutes and then the following were added to the tubes: 4 µl of

5x first strand buffer (Invitrogen), 0.01 µmoles of dNTPs (Invitrogen), 0.02 µmoles of DTT (Invitrogen), 10U of RNase Out inhibitor (Invitrogen) and 100U of SuperScript III reverse transcriptase (Invitrogen) and incubated at the following conditions: 25°C for 15 min, 42°C for 50 min, 70°C for 15 min and 4°C hold temperature. The final volume for this reaction was 20µl.

The PCR amplification of targeted viral sequences in the cDNA was performed in a 25-µLreaction containing: 12.5 µl of Amplitaq Gold 360 PCR master mix (Applied Biosystems USA), 0.5µl of 50 picomoles each of forward and reverse primer, 2 µl of the cDNA and 9.5 µl of DEPC treated water. Samples were first tested using alphavirus family primers (VIR2052F5'-TGG CGC TAT GAT GAA ATC TGG AAT GTT-3' and VIR2052R 5'-TAC GATGTT GTC GTC GCC GAT GAA-3') (Eshoo et al., 2007). Samples testing positive with alphavirus family primers were further tested with chikungunya specific primers (7028 forward (5'-GCGCGGCCTTCATCGGCGACTAC-3') and 8288 reverse (5'CCAGGTCACCACCGAGAGGG-3') (Sang et al., 2008), in all the PCR reactions, appropriate positive control cDNA (Lamu33, HQ456255.1) and a negative control (nuclease free water) were included. Electrophoresis of the amplified DNA products was done on a 1–2% agarose gel in 1% Tris-borate EDTA buffer stained with ethidium bromide. A ultra-violet (UV) trans-illuminator was used to visualize the PCR product bands and images recorded using a gel photo imaging system.

3.7 Virus quantification

Quantification of CHIKV was performed by plaque assay. Ten-fold serial dilutions of the amplified CHIKV was carried out and inoculated in 6 well plates containing confluent Vermonolayers of Vero cells as described by Gargan(Gargan *et al*., 1983). This was grown in MEM, with Earle's salts and reduced $NaHCO_3$, supplemented with 10% heat-inactivated FBS, 2% L-Glutamine, and 2% antibiotic/antimycotic solution with 10,000 units penicillin, 10 mg streptomycin and 25µg amphotericin B per ml and incubated at 37°C in 5% CO_2 overnight. Each well was inoculated with 100 µl of virus dilution, incubated for 1 hour with frequent agitation/rocking to allow adsorption. The infected

cells were maintained using 2% methylcellulose mixed with 2X MEM (GIBCO® Invitrogen corporation, Carlsbad, California) and incubated at 37°C with 5% CO_2 for 4 days then fixed for 1 hour with 10% formalin, stained for 2 hours with 0.5% crystal violet and the plaques counted and calculated to quantify the virus using the formula:

$$\frac{\text{\# of plaques}}{\mathrm{d} \times \mathrm{V}} = \mathrm{PFU/ml}$$

where # is the number, d is the dilution factor and V is the volume of diluted virus added to the wells

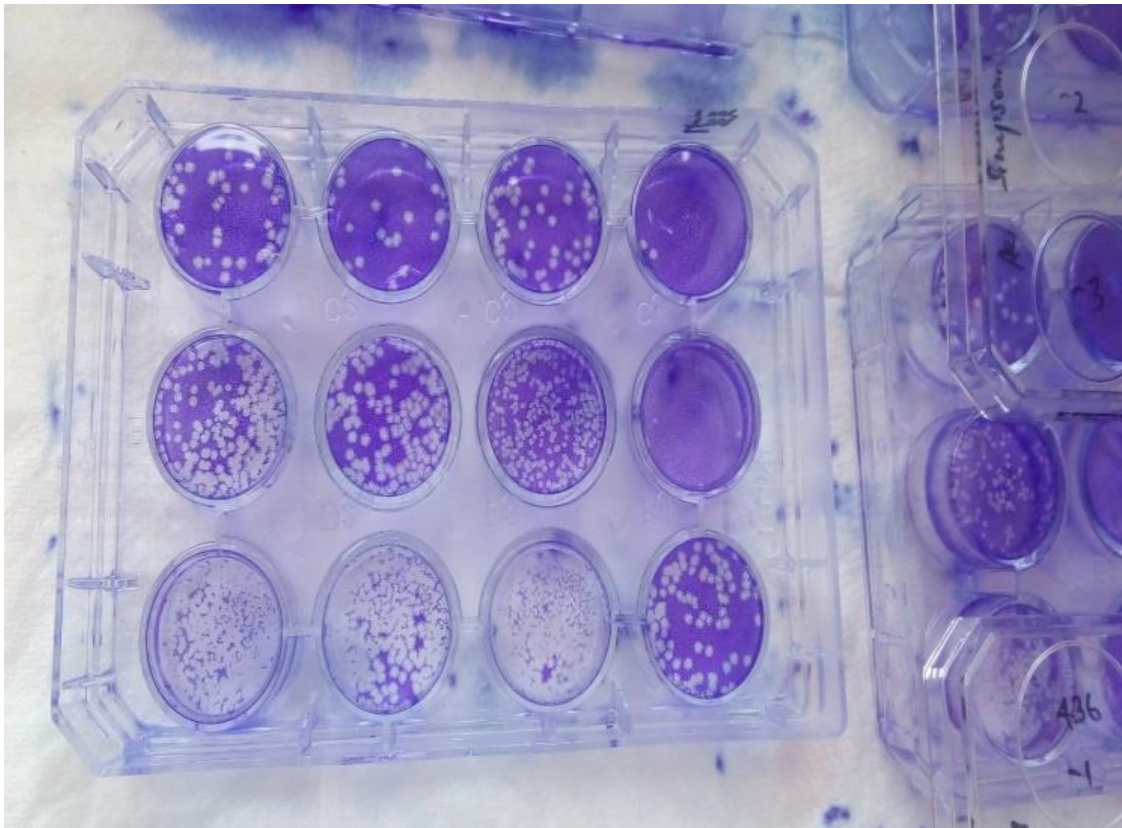


Plate 3.2: Twelve well plate showing chikungunya virus plaques forming unit on serial diluted viral titres

3.8 Sampling and preparation of mosquitoes for assays.

The wild filial generation (F_0) and first generation (F_1) generations of females *Ae. bromeliae* and *Ae. vittatus* respectively were deprived of glucose for 24 hours before exposure to the infectious blood meal, using an artificial membrane feeding system (hemotek). The virus/blood mixture was put in membrane feeders covered with freshly prepared mice skin, and maintained using the hemotek system which employs an electric heating element that maintains the temperature of the blood meal at 37°C. Batches of 50–100 female mosquitoes aged 4–5 days were fed on virus blood mixture at a ratio of 1:1 CHIKV isolate and defibrinated sheep blood using a hemotek feeding system for 60 minutes. Only fully engorged mosquitoes were transferred to 4-litre plastic cages (15–30 mosquitoes/ cage) with a net on top and maintained with 10% glucose at 28-30°C, relative humidity of 70-80%, and 12:12 hour L: D photoperiod, while the non-engorged mosquitoes were destroyed. Mosquito mortality was monitored in the cages by removing and counting dead mosquitoes daily. The experiment was done in three replicates to obtain the sufficient sample size for a good statistical power. The non ergorged and male mosquitoes were grouped in pools of up to 25 mosquitoes and were hogeninized on the homogenization media using the bead ruptor, the homoginates were used as negative control for the plaque assay.

3.9 Infection and dissemination rate

On day 5, 7 and 10 post infection (dpi), a representative samples (at least 30%) of the orally exposed mosquitoes were picked, cold knocked down on -200c fridge to immobilize them and carefully decapitated with the legs/wings and bodies placed into separate 1.5mL microfuge tubes (Eppendorf). Each mosquito body was placed separately in a well labelled 1.5ml tubes containing 1000 µl of homogenization media (HM), made of MEM, supplemented with 15% FBS, 2% L-Glutamine, and 2% antibiotic/ antimycotic. Mosquito bodies were homogenized using a Mini bead beater (BioSpec Products Inc, Bartlesville, OK 74005 USA) with the aid of a copper bead (BB-caliber airgun shot) and clarified by centrifugation at 12,000 rpm (Eppendorf centrifuge 5417R) for 10 minutes at 4˚C. The

supernatant were inoculated in Vero cell lines in 12 well plates, grown in MEM, supplemented with 10% FBS, 2% L-Glutamine and 2% antibiotic/ antimycotic. One hundred microliters of the appropriate dilutions of the abdominal homogenates was added to each of ten wells of the 12-well plate to infect the cells and the remaining two wells were used for controls, for negative control we used male mosquitoes from the study vectors comprising of a pool of 25 mosquitoes. The plates were incubated at 37 °C in 5% CO_2 incubator with frequent agitation after every 15 minutes for 1 hour to allow for virus adsorption. The infected cell monolayers were then overlaid with 2.5% methylcellulose supplemented with 2% FBS, 2% L-Glutamine and 2% antibiotic/ antimycotic and incubated at 37°C in 5% CO_2. On day 4, plates were fixed for 1 hour with 10% formalin, and stained for 2 hours with 0.5% crystal violet, washed on running tap water, dried overnight and the plaques observed on a light box. The CHIKV positive bodies were used to determine the infection rates. For each positive abdomen, corresponding legs were homogenized and their infection status determined as described above for the abdomens. Plaques were counted and calculated to determine the viral titer. If the virus was detected in the mosquito's body but not in the legs, the mosquito was considered to have a non-disseminated infection limited to the midgut. Detection of virus in the body and legs was considered as evidence of successful infection and dissemination respectively (Turell *et al*., 1992).

Plate 3.3: Mosquito feeding experimentation using Hemoteck membrane feeder the feeder uses mice skin as a membrane to allow the mosquitoe obtain infectious blood meal from the mice skin.

3.10 Test for transmission rate

After exposing the mosquitoes to the infectious blood meal only the engorged mosquitoes were picked, placed into new cages, incubated under the insectary conditions and maintained with10% sucrose. On 5, 7 and 10 days post infection (dpi), they were sucrose-starved and deprived of water for 16 hours, cold knocked down in -20°c fridge for 40 seconds before the legs and wings from each of them were carefully removed and placed on sticky tape. Individual mosquito proboscis was inserted into a capillary tube containing 10-20ul of HM. The mosquitoes were allowed to expectorate saliva for 30 minutes and the media containing saliva was then expelled into a cryovial containing 200ul of MEM and stored at -80°C until tested. A volume of 80µl of the saliva sample was inoculated into each well of a 24-well plate containing confluent Vero cell monolayers. Plates were incubated for 1 hour to allow for adsorption, with frequent agitation/rocking. The infected

cells were maintained using maintenance media (1 ml per well) and incubated at 37˚C with 5% CO_2. Plates were observed for 7 days and the supernatant of wells showing CPE were harvested and virus quantified by plaque assay as discribed above. Plaques were counted and calculated to quantify the virus

Plate 3.4: Individual mosquito proboscis inserted into a capillary tube to allow salivation.

3.11 Data Management and Analysis

The data was entered in a laboratory work sheet as hard copy and spread sheet in a soft copy and stored in backed flash disk. Three parameters describing vector competence were determined: infection (number of infected mosquito bodies per 100 mosquitoes orally exposed and tested), dissemination (number of mosquitoes with positive legs per 100 mosquitoes infected) and transmission rates (number of mosquitoes with positive

saliva per 100 mosquitoes with disseminated infection). Test of proportions were used to get the infection, transmission and dissemination rates with their 95% CI. Statistical analyses were conducted in SAS (SAS Institute,Cary, NC). Chi-square test with or without Yates' correction or Fisher's exact test were used to analyze significant differences ($P < 0.05$) in infection, dissemination, and transmission rates for each EIT. Titers of bodies and legs were log-transformed [log (x +1)] prior to analysis to normalize data. Analysis of variance (ANOVA, PROC GLM) was carried out separately for each body part to show differences in titers between EITs and species. Statistical significance was considered for $P < 0.05$.

3.12 Ethical Considerations

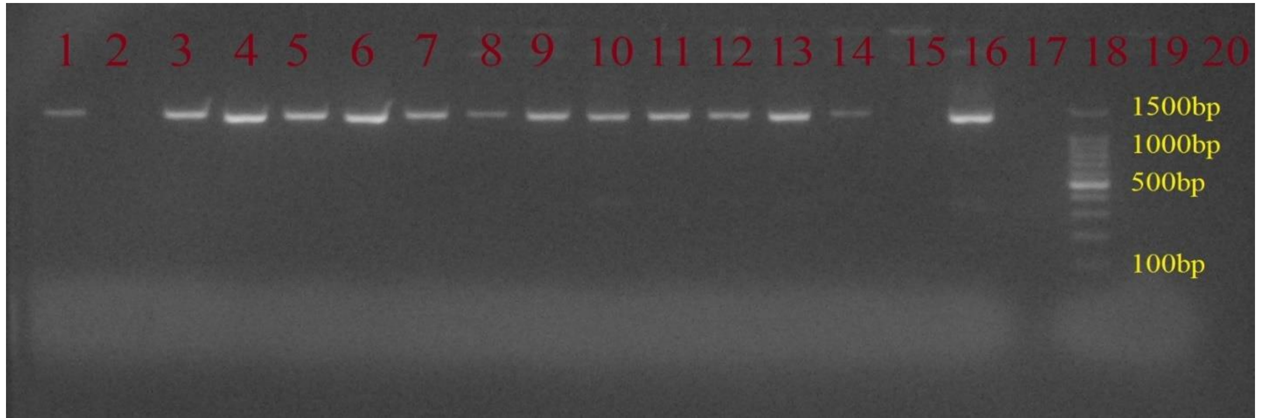
Scientific and ethical Approval to carry out this study was obtained from the KEMRI Scientific Ethical Review Unit (SERU) (KEMRI/SERU/CVR/002/3449) (APPENDIX 3). The animal use component was reviewed and approved by KEMRI Animal Care and Use Committee (ACUC) (KEMRI/ACUC/01.05.17) (APPENDIX 2).. The KEMRI ACUC adheres to national guidelines on the care and use of animals in research and education in Kenya enforced by National Commission for Science, Technology and Innovation (NACOSTI). The Institute has a foreign assurance identification number F16-00211 (A5879-01) from the Office of Laboratory Animal Welfare (OLAW) under the Public Health Service and commits to the International Guiding Principles for Biomedical Research Involving Animals.

CHAPTER FOUR

RESULTS

4.1 RT-PCR results

As a quality control and quality assurance requirement the reverse transcriptase polymerase chain(RT-PCR) test was done to confirm the test results were true for the test. The male and non ergorged females were used as a negative control for the test while the kit positive control were used as a positive control. The test showed the negative and positive test were all corresponding to the plaque test results as shown (fiqure 4.1)



Lane 1: Positive control

Lane 2: Negative control

Lane 3-14, 16: Chikungunya virus positive sample used for the study

Lane 15: Male mosquitoes homogenates used in the study as negative control for plaque assay

Lane 17: Non engorged female mosquitoes homogenates used in the study as negative control for plaque assay

Lane 18: Molecular marker

Lane 19, 20: Empty wells

Figure 4.1: Agarose gel electrophoresis demonstrating the 1150 base pair corresponding to the generated RT-PCR for Chikungunya virus.

4.2 Species breeding preference

All the larvae and pupae that were collected from plant leave axils were *Ae. bromeliae* this shows that the *specie* prefers natural habitant as it breeding preference while over

70% of larvae and pupae that were collected from rock pools and tree holes were *Ae. vittatus* although a few species were collected from artificial containers. The mosquito species that were used in this study and the breeding habitats are shown in the table below (Table 4.1).

Table 4.1: Mosquito species and their preferred breeding habitats.

Mosquito species	**Habitat type**	**Breeding sites**	**Date of collection**	**Stage**
Ae. simpsoni bromeliae	Peridomestic	Banana leaf axils	July 2017	Larvae and pupae
		Arrow root leaf axils	July 2017	Larvae and pupae
		Flower axils	July 2017	Larvae and pupae
Ae. Vittatus	Peridomestic	Rock pools/holes	May/2017	Eggs, larvae, pupae
	Forest	Rock pools/holes	May/2017	Eggs, larvae, pupae
		Tree holes	May/2017	Eggs, larvae, pupae

4.3 ***Ae. bromeliae*** **and** ***Ae. vittatus*** **infection, dissemination and transmission potential.**

The analysis of different post-inection time points showed that ae. bromeliae and ae. vittatus became infected jfor the ESCA lineage of chikungunya virus,The feeding success rate of the two mosquito species on infected blood meal was high; and ranged from 70-80% for *Ae. bromeliae* and 40-50% for *Ae. vittatus*. The blood meal titres were determined before and immediately after mosquito exposure, the infection rate for Kilifi and West

Pokot mosquitoes were measured from a total of 311for *Ae. bromeliae* (110 on 5 dpi, 101 on 7 dpi and 100 on 10 dpi) and 204 for *Ae. vittatus* (69 on 5 dpi, 69 on 7 dpi and 66 on10 dpi). Both species were susceptible to chikungunya virus infection with average infection rates of 37% and 79% for *Ae. bromeliae* and *Ae. vittatus* respectively. *Ae. vittatus* had high midgut infection rate but with no significant difference between the extrinsic incubation periods. The overall dissemination rate was high for *Ae. vittatus* with more than 46% of the mosquitoes with midgut infection having disseminated infection. *Ae. bromeliae* had moderate midgut infection rate on 5 and 7dpi. Overall *Ae. bromeliae* showed relatively low dissemination with 34% of those with midgut infection having disseminated infection (Table 4.2). The infection rates within species were different but not significantly so between different EIPs; but were significant between species throughout the three EIPs.

Table 4. 2: Infection, dissemination and transmission rates of mosquitoes orally infected with CHIKV (Infectious blood meal = 106.4 PFU/mL).

			Day 5		Day 7		Day 10
		n	Infection rate% (95%CI)	n	Infection rate% (95%CI)	n	Infection rate% (95%CI)
Percent of infection	*Ae. bromeliae*	45	40.9 (31.6 - 50.7)	44	43.6 (33.7 - 53.8)	26	26.0 (17.7 - 35.7)
	Ae. vittatus	56	81.2 (69.9 - 89.6)	54	78.3 (66.7 - 87.3)	52	78.8 (67.0 - 87.9)
	p value		**<0.001**		**<0.001**		**<0.001**
Percent of disemination	*Ae. bromeliae*	12	26.7 (14.6 - 41.9)	16	36.4 (22.4 - 52.2)	11	42.3 (23.4 - 63.1)
	Ae. vittatus	26	46.4 (33.0 - 60.3)	23	42.6 (29.2 - 56.8)	26	50.0 (35.8 - 64.2)
	p value		**0.042**		0.531		0.521
Percent of transmission	*Ae. bromeliae*	5	41.7 (15.2 - 72.3)	5	31.3 (11.0 - 58.7)	6	54.5 (23.4 - 83.3)
	Ae. vittatus	11	42.3 (23.4 - 63.1)	11	47.8 (26.8 - 69.4)	9	34.6 (17.2 - 55.7)
	p value		0.97		0.301		0.259

4.4 *Ae. bromeliae* and *Ae.vittatus* susceptibility to CHIKV midgut infection.

Aedes vittatus was highly susceptible to CHIKV with infection rates of 81%, 78%, and 79% on 5, 7 and 10 dpi respectively compared to *Ae. bromeliae* which was moderately susceptible with infection rates of 44%, 41% and 26% on 5, 7 and 10 dpi respectively (figure 4.2).This show the variant on the midgut influences susceptibility to virus infection, *Ae. bromeliae* has a strong midgt barrier compared to *Ae. vittatus* Infection rates for *Ae. vittatus* was higher relative to that of *Ae. bromeliae*. Statistical significant difference was observed for infection rates on 5 dpi for *Ae. bromeliae* (40.9%, 95% CI [31.6-50.7%]) and *Ae. vittatus* (81.2%, 95% CI [69.9-89.6%]) $p < 0.001$; Day 7 for *Ae. bromeliae* (43.6%, 95% CI [33.7-53.8%]) and *Ae. vittatus* (78.3%, 95% CI [66.7-87.3%]) $p < 0.001$; and Day 10 Day 5 for *Ae. bromeliae* (26.0%, 95% CI [17.7-35.7%]) and *Ae. vittatus* (78.8%, 95% CI [67.0-87.9%]) $P < 0.001$.

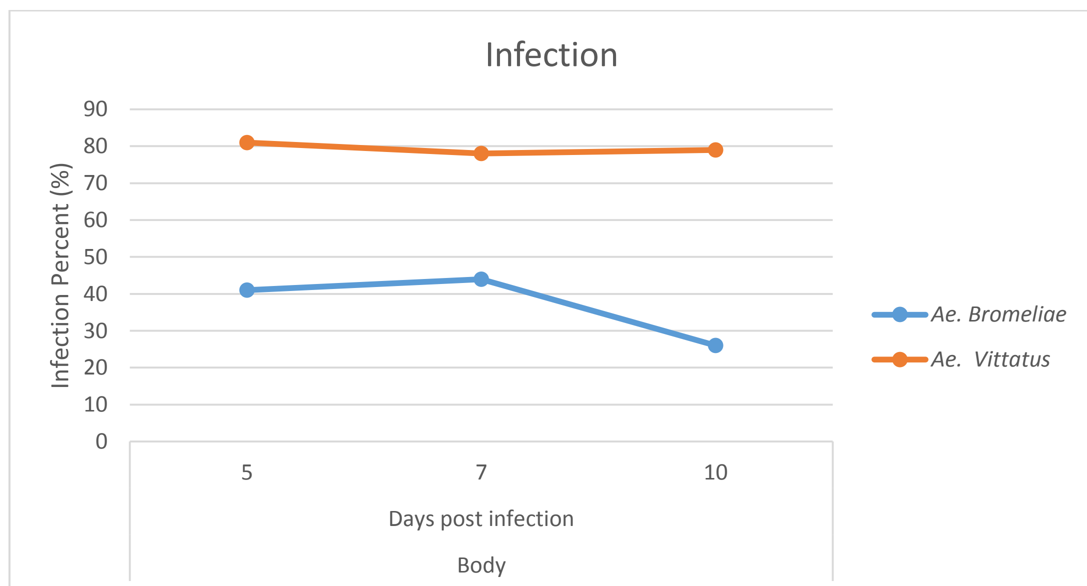


Figure 4.2: Proportion of Kilifi Ae. simpsoni bomeliae and West Pokot Ae. vittatus infected with CHIKV at day 5, 7 and 10 of post infection.

4.5 *Ae. bromeliae* and *Ae. vittatus* susceptibility to CHIKV dissemination infection.

Dissemination rates for *Ae. vittatus* were higher relative to those of *Ae. bromeliae*. However, statistical significant difference was observed for 5 dpi, *Ae. bromeliae* (26.7%, 95% CI [14.6-41.9%]) and *Ae. vittatus* (46.4%, 95% CI [33.0-60.3%]) $p < 0.001$. Viral dissemination was observed as early as 5-7 dpi for both species. The proportion of disseminated infection for *Ae. bromeliae* increased significantly with increase in the number of days post infection with higher rate on day 10 (43%). *Ae. bromeliae* had dissemination rate of 26%, 36% and 43% at 5, 7 and 10 days post infection (figure4). *Aedes vittatus* showed no significant difference on dissemination rates between the difference dpi although higher dissemination rate was observed on 10 dpi (50%). *Aedes vittatus* had disseminated infection rates of 46%, 43% and 50% at 5,7 and 10 dpi, respectively, but these differences were not statistically significant (chi-square test, $p>0.05$) (figure 4.3). The overall data shows that 114 out of 277 mosquitos with midgut infection disseminated the virus to the legs, the *Ae. vittatus* population from West Pokot county had higher dissemination rate (46%), than the *Ae. bromeliae* (34%) population from Kilifi county.

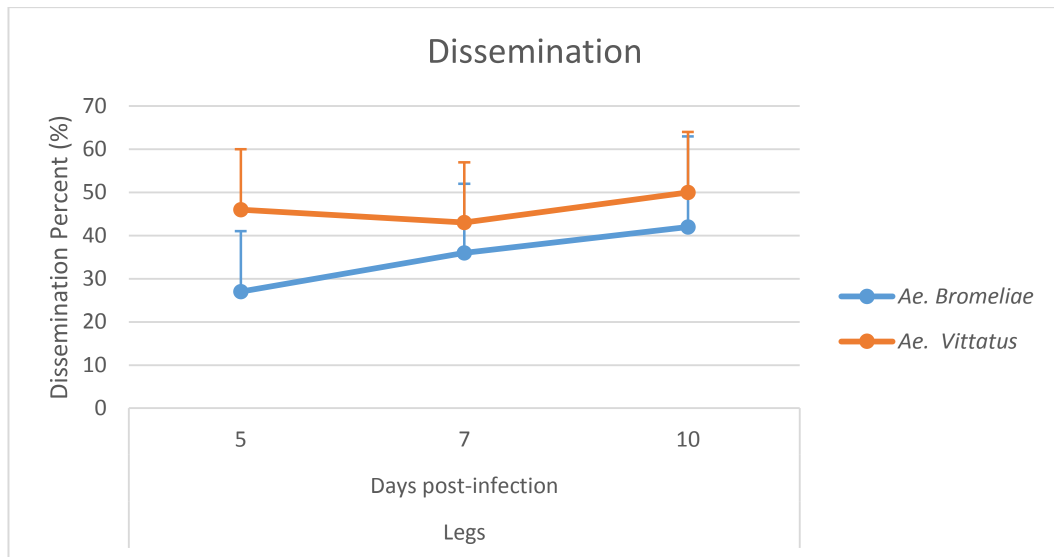


Figure 4.3: Proportion of Kilifi Ae. bromeliae and West Pokot Ae. vittatus infected mosquitoes with dissemination at day 5, 7 and 10 of post infection.

4.6 *Ae. bromeliae* and *Ae.vittatus* susceptibility to CHIKV transmission.

Both species were able to transmit the virus as early as 5dpi. Transmission rate for *Ae. bromeliae* was higher on day 10 (55%) compared to other days post infection . *Ae. vittatus* had higher transmission rate on day 7 (48%) which significantly declined on day 10 (35%) of post infection (figure 4.4). The overall data of both Kilifi and West Pokot mosquitoes population shows that 46 out of 114 (40%) were able to transmit the virus. Although *Ae. vittatus* had higher infection and dissemination, there was no significant difference on overall transmission on both vectors (*Ae. vittatus* 41% and *Ae. bromeliae* 41%)

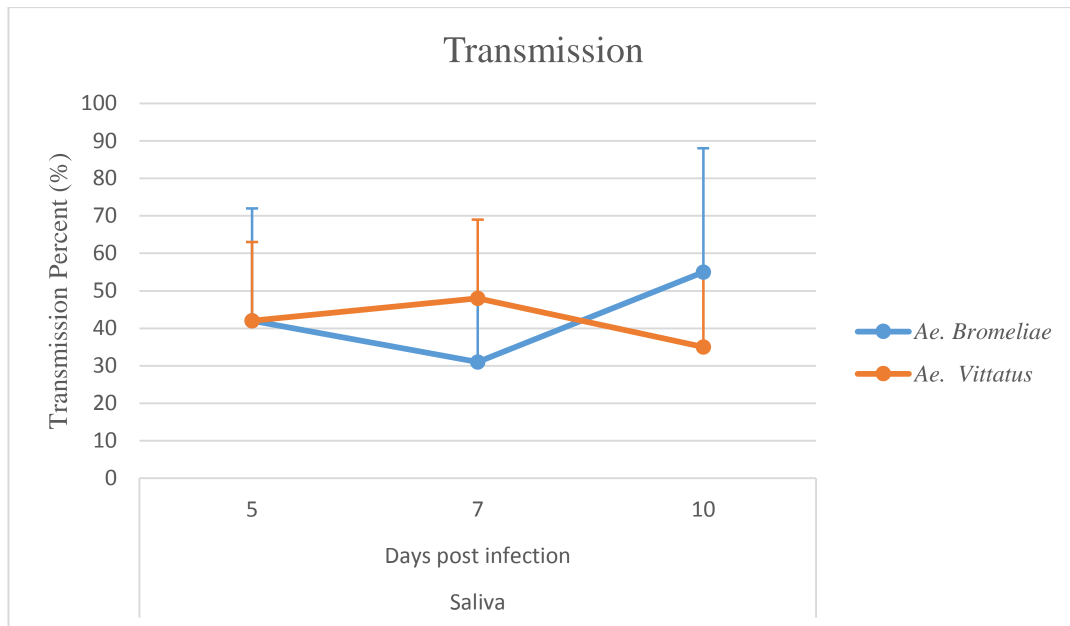


Figure 4.4: Proportion of Kilifi *Ae. bromeliae* and West Pokot *Ae. vittatus* infected (A) and disseminated (B) and transmission infection (C) with CHIKV at 5, 7 and 10 days post infection.

Ae. bromeliae dissemination efficiencies increased with increase in the number of days post infection as shown(Figure 4.5), *Ae vittatus* had high rate of dissemination efficiencies on 7 dpi Overall transmission rates for *Ae. vittatus* was higher relative to that of *Ae. bromeliae* though no statistical significance was observed (chi-square test, P>0.05).

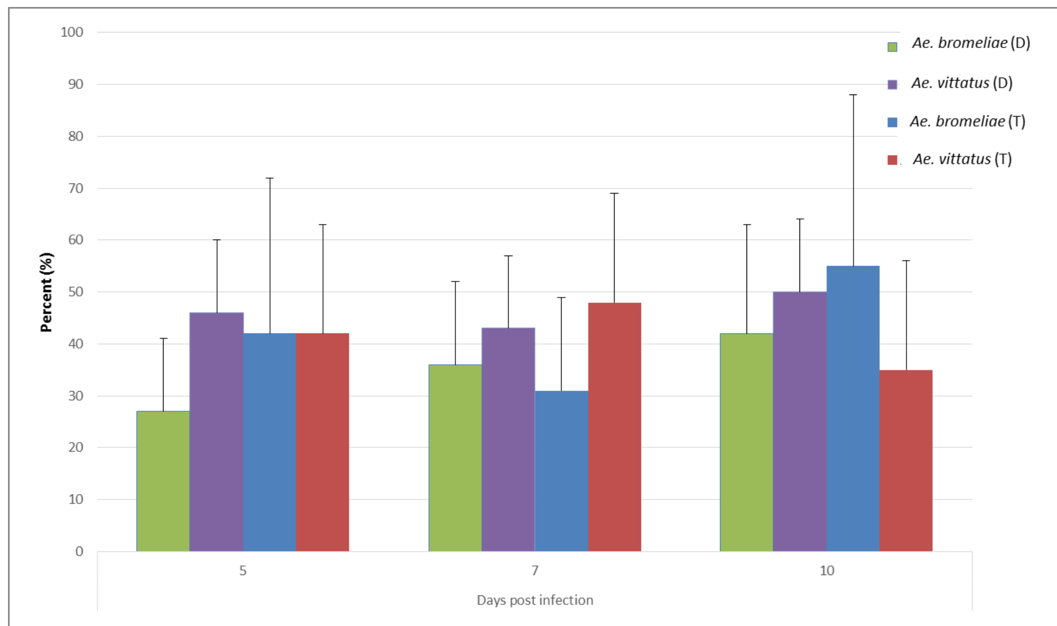


Figure 4.5: Progression trends of CHIKV dissemination and transmission efficiencies in *Ae. bromeliae* and *Ae. vittatus.*

4.7 Chikungunya virus replication dynamics in the analyzed mosquito populations.

Ae. bromeliae and *Ae. vittatus* were analysed to asses viral titers in bodies and legs plus wings by titration in Vero cells. *Ae. bromeliae* bodies showed mean viral titers of 5.0± 0.33 $\log_{10}$ PFU/mL, 5.3± 0.34 $\log_{10}$ PFU/mL, 5.3± 0.45 $\log_{10}$ PFU/mL (mean ± ΔS) PFU/ML at 5,7,10 days of post infection respectively, the titers increased with increase in the days of post infection Indeed the mean CHIKV titers in the bodies increased progressively reaching a value of 5.3 ± 0.45 $\log_{10}$ PFU/mL at the 10th days post infection (Figure 4.6). The viral presence in the legs was detected as early as the 5 day of post infection with a titer of 4.0± 0.58 $\log_{10}$ PFU/mL and at 4.3± 0.52 $\log_{10}$ PFU/mL, 4.3± 0.62 $\log_{10}$ PFU/mL on day 7 and 10 dpi respectively. *Ae. vittatus* bodies showed mean viral titers of 5.7± 0.32 $\log_{10}$ PFU/mL, 5.8± 0.32 $\log_{10}$ PFU/mL, 4.9± 0.31 $\log_{10}$ PFU/mL (mean ± ΔS) PFU/ML at 5,7,10 days of post infection respectively (Figure 4.6). The viral presence in the legs was detected as early as the 5 day of post infection with a titer of 3.6± 0.37 $\log_{10}$ PFU/mL and at 4.4± 0.441 $\log_{10}$ PFU/mL, 4.2 ± 0.40 $\log_{10}$ PFU/mL on day 7 and 10 dpi respectively. Our results highlighted that in all *Ae. bromeliae* and *Ae. vittatus* populations, CHIKV was able to

infect mosquitoes and replicate over time, disseminating in wings and legs and reaching the salivary glands. There is no significance difference in both vectors for infection and dissemination mean titers.

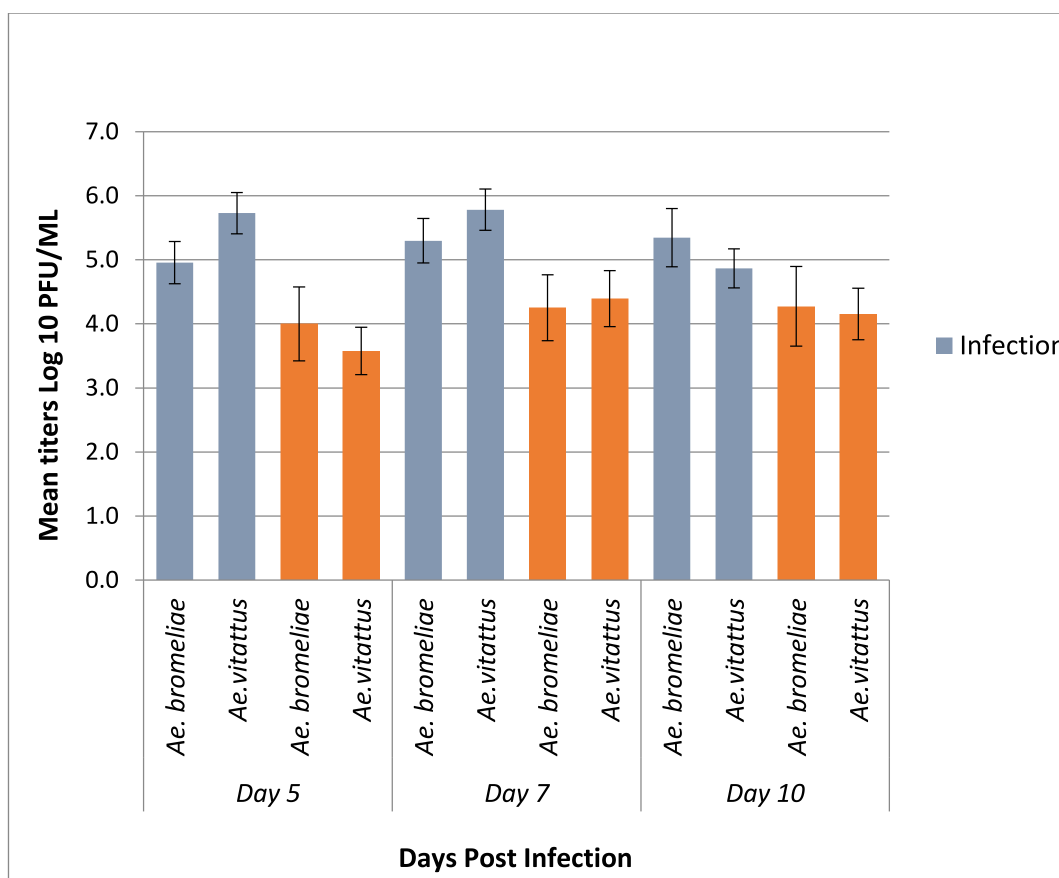


Figure 4.6: Chikungunya virus replication in Ae. bromeliae and Ae. vittatus. Comparisons of CHIKV mean titer. Infected Ae. bromeliae and Ae. vittatus females was calculated by serial titration on VERO cells

Based on the titers detected for each species, the mosquitoes that were susceptible to infection and failed to disseminate the virus had titers at least a log lower than mosquitoes which were susceptible and had disseminated the virus. In general, viral dissemination only occurred when body titers were $\geq 10^5$ for both strains**,** *Ae. bromeliae* had a midgut infection barrier that was stronger than that of *Ae. vittatus* but both species were similar in their ability to transmit the virus . However, there was no difference in leg titers observed, for those that did, or did not, transmit the virus (Table

4.3). No statistical difference for mean titers for the *Ae. bromeliae* and *Ae. vittatus* observed for all the dpi (chi-square test, $p>0.05$)

Table 4.3: Mean body and leg titers for Ae. vittatus and Ae. bromeliae exposed to chikungunya virus.

species	Non disseminated	Disseminated [b]		Mean leg titer [c]	
	Body titers [a]	Body titer	Leg titer	N.T	TRANS
Ae. bromeliae	$10^{4.9}$	$10^{5.8}$	$10^{4.1}$	10^{4}	$10^{4.3}$
Ae. vittatus	$10^{5.4}$	$10^{5.9}$	10^{4}	$10^{3.9}$	$10^{4.4}$

[a] Mean body titer for infected mosquitoes with negative legs (PFU/specimen)

[b] Mean titers for infected mosquitoes with positive legs(PFU/specimen)

[c] Mean leg titers for virus-positive legs with negative saliva (N.T. = non transmitters) and those with positive saliva (Trans. = transmitters) (PFU/specimen).

CHAPTER FIVE

DISCUSSION

5.1 Discussion

This is the first study to determine the ability of *Ae. bromeliae* and *Ae. vittatus* mosquito populations from Kenya to transmit the ECSA lineage of CHIKV. This study has demonstrated that the two are laboratory competent vectors of ECSA lineage of CHIKV. The past outbreak of chikungunya in Africa, America, Asia and Europe (Angelini *et al.*, 2007; Chretien & Linthicum, 2007; Zeller *et al.*, 2016a), clearly demonstrate the potential of the disease to spread to new areas and cause massive epidemics. The risk of importation of CHIKV to new areas is due to international and local travels from epidemic areas and exporting of the infected vectors to new areas where there are susceptible people and competent vectors (Agha *et al.*, 2017c; Richards *et al.*, 2010). The full competence of a vector is not only determined by the ability of the vector to get infected but also by its ability to transmit pathogen (Moncayo *et al.*, 2004). In this study we determined the ability of the two mosquito populations to get infected, disseminate and transmit the virus. The midgut infection and escape barrier and salivary gland barriers are the most important determinant of the vector with regard to viral replication and transmission in a vector (Anderson & Rico-Hesse, 2006).

The CHIKV titers ($10^{6.4}$ PFU/ml) used to infect mosquitoes in this study, are almost similar to published viremia levels associated with human infections (often $>10^5$ PFU/mL of blood) in nature (R. S. Lanciotti *et al.*, 2007). It has also been shown that a titer of 10^4 PFU/ml in monkeys was sufficient to infect mosquitoes (Turell *et al.*, 1992). The findings shows that the two mosquito species are susceptible and have ability to transmit CHIKV. Although all mosquito species tested had ingested infectious blood meals, not all mosquitoes got infected and not all that were infected had the virus disseminated, this shows that other factors such as midgut escape barrier affect the replication and dissemination of the virus in a mosquito (Ciota & Kramer, 2013). More than 27% of mosquitoes per population were able to disseminate CHIKV

after crossing the midgut infection and escape barriers, epithelial cells that allows viral replication and release of virions.

Ae. bromeliae population from Kilifi county breeds preferably on banana axils and flower axils, all the larvae and pupae collected from the banana leaf axils were *Ae. bromeliae.* The preference is similar to other studies where high abundance of *Ae. bromeliae* were observed breeding in water holding leaf axils of bananas and arrow roots (Mukwaya *et al*., 2000). *Ae. bromeliae* population had moderate midgut infection which ranged from 26-44% across the different days post infection. Virus infection in the midgut was detected as early as 5 days post infection, this is similar to previous studies which showed that the mosquito bodies infection with CHIKV in East Africa ranges from 2 -9 days (Rudolph *et al*., 2014). Infection and dissemination in the early days of post infection is as result of CHIKV's ability to replicate faster at high temperature (Alto *et al*., 2018; Mbaika *et al*., 2016). This suggests that these mosquitoes have weak midgut infection and escape barriers. *Ae. bromeliae* had higher transmission rate on10 dpi compared to *Ae. vittatus* which had higher transmissions on day 7 dpi , this may suggest that *Ae. bromeliae* requires more days for the virus to infect the salivary glands and eventually transmit to a susceptible host.

Ae. vittatus from breeds mostly on rock pools/holes, tree holes as demonstrated by the over 70% of the total collection. Breeding of *Ae. vittatus* in rock pools and tree holes has been previously demonstrated (Diagne *et al*., 2014; Diallo *et al.*, 2012a; Diallo *et al*., 1999). The study showed that the West Pokot population of *Ae. vittatus* have the potential to transmit CHIKV as it has been demonstrated in other studies (Diagne *et al*., 2014). The study showed that *Ae. vittatus* midgut infection and dissemination rate within the 5dpi were relatively high suggesting the presence of weak midgut infection (MIB) and escape barriers (MEB). Therefore, West Pokot *Ae. vittatus* population is a more efficient in transmitting CHIKV and indicates a potential risk if the virus is introduced in the area . Early dissemination observed in *Ae*. *vittatus* within 5 dpi at a relatively high rates indicates high susceptibility of this species, Similar results have also been observed in previous studies elsewhere (Diagne *et al*., 2014).

The study demonstrated that not all *Ae. bromeliae* and *Ae. vitattus* are capable of transmitting the CHIKV via capillary feeding this shows dissemination is dependent on the midgut infection (Turell *et al*., 1996). Under the conditions of this test, not all *Ae. bromeliae* and *Ae. vitattus* with body infections and infections disseminated to the legs are infectious, that is capable of transmitting CHIKV in saliva. However, the current study only provides estimates of *in vitro* transmission, which does not necessarily represent the amount of virus inoculated in a host during natural blood feeding, and there is a limitation with regard to the sensitivity of the assay that must be considered. We found no differences in leg or saliva titers between *Ae. bromeliae* and *Ae. vitattus* regardless of EIT, showing that virus titers in both species level off at a similar rate. Despite the two species being exposed to the same virus titers, *Ae. vittatus* showed high infection and dissemination rates compared to *Ae. bromeliae.* This may be due to other intrinsic factors such as varying strength of midgut infection barrier(MIB) and midgut escape barrier (MEB) that individually affect the susceptibility of different mosquito species to infections (Moreira *et al*., 2009). While *Ae. vittatus* had higher midgut infection than *Ae. bromeliae*, the two species had no significant difference on transmission regardless of the incubation period. This suggests that the salivary gland barrier is independent of the midgut infection and determined by the ability of the virus to penetrate into the saliva glands and be secreted into the saliva (Kramer *et al*., 1981). For both species, the mosquitoes which had a higher viremia in their infected legs were able to transmit the virus by capillary method. Although this method is not accurate in testing transmission, the percentage transmission observed showed the method can be used to test for transmission on viruses which have no documented animal models for such experiments. Mosquitoes usually secrete less virus into a capillary tube than when feeding on an animal (Styer *et al.*, 2007) and transmission rates are often lower when they are determined by collection of saliva as compared to allowing the mosquito to feed on a susceptible animal (Akhter *et al*., 1982). Therefore, failure to detect CHIKV in the saliva collected in a capillary tube does not mean that the mosquito would not have transmitted the virus by bite if it fed on a susceptible human. In this case, our transmission rates should be considered as minimum transmission rates. Although *Ae. vittatus* and *Ae. bromeliae* from Kenya are efficient laboratory vectors, their potential role in CHIKV

transmission depends on other factors in relation to mosquito ecology such as densities, survival, longevity, anthropophily and duration of gonotrophic cycles, which have been shown to interfere with transmission and maintenance of CHIKV. Vector-virus interactions contributing to vector competence are complex (Richards *et al*., 2009), hence additional populations, virus doses, environmental conditions, and factors would need to be tested in order to begin to evaluate the relative importance of these mosquitoes in the natural CHIKV transmission cycle. Surveillance should be done continuously to avoid introduction of the virus or for early detection of CHIKV in this territories, through viremic travelers.

5.2 Study limitation

Due to lack of equipment and supplies quality control for viral RNA extracted for the RT-PCR was not carried out to quantify viral RNA.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusion

1. This study demonstrated that *Ae. vittatus* and *Ae. bromeliae* populations in West Pokot and Kilifi respectively have got different preferred breeding areas as demostrrated in the study.
2. The two vectors were laboratory competent vectors of ECSA lineage of CHIKV since they were able to infect the midgut, dessiminate the infections to the legs.
3. The two *species* were able to transmit the virus through the saliva via capillary feeding hence making them competent vectors for the CHIKV.
4. . The finding indicates there is high potential for CHIKV transmission in the study areas to occur should the virus find its way there through travel or introduction via sylvatic host..

6.2 Recommendation

1. Community based vector control initiative should be done in the area to minimize vector densities. Residents should be able to monitor mosquito breeding's areas and drain water to destroy their breeding habitants
2. Futher studies to determine requlatory proteins in midgut infection barrier for the different in susceptability to infection and dissemination and non susceptabily and non disseminated infections.
3. Public education on the epidemiology of CHKIV,
4. Recommend more studies to determine whether the two vectors can maintain the virus through transovarial transmission.

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APPENDICES

Appendix I: Immature Mosquito Survey Sampling Tool

Immature mosquito survey sampling tool

Inspectors:…………………………………………… Area……………………………………………site……………………………………Date…………………………

House No./ ID	Container type	Number of containers							
Indoors	**Artificial containers**	Larvae -ve	Larvae +ve	Pupae -ve	Pupae +ve	Total wet container	Total wet container	Total +ve container	Total container with pupae
	Jerry can								
	Bucket								
	Plastic drum								
	Clay pots								
	Plastic basins								
	others								
	others								
Outdoor	**Artificial containers**								
	Jerry can								
	Bucket								
	Plastic drum								
	Clay pots								
	Plastic basins								
	others								
	others								
	Natural containers								
	Coconut shells								
	Banana axils								
	Tree holes								
	others								
	others								
Peri domestic	Banana axils								
	Tree holes								
	Rock pools								
Forest	Banana axils								
	Tree holes								
	Rock pools								

Appendix II: Acuc Approval Letter

KENYA MEDICAL RESEARCH INSTITUTE

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

KEMRI/ACUC/ 01.05.17 **3rd May 2017**

Francis Mulwa
CVR

Mulwa,

RE: Animal use approval for "Determination Of Vector Competence Of Aedes Simpsoni Mosquito Complex From Kilifi (Coastal) and Aedes Vittatus From West Pokot Region For Chikungunya " protocol

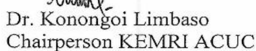
The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that all the use of laboratory mice is justified in achieving some of the study objectives and issues raised earlier have been adequately addressed.

Approval is granted for a period of one year starting from when the SERU approval will be obtained. If you still intend to handle laboratory mice after the period covered by this initial approval, you are required to submit an application for continuing approval to the ACUC 1 month prior to the expiry of the initial SERU approval. In addition, the committee expects the study to provide an annual report on the progress of animal use simultaneously with the annual continuing review report to SERU.

The committee expects you to adhere to all the laboratory animal handling procedures as described in the protocol.

The committee wishes you all the best in your work.

Yours sincerely,



Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

KENYA MEDICAL RESEARCH INSTITUTE
★ 03 MAY 2017 ★
ANIMAL CARE AND USE COMMITTEE
Signature: ....

In Search of Better Health

Appendix III: Ethical Review Committee Approval Letter

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

KEMRI/RES/7/3/1 **May 15, 2017**

TO: **FRANCIS MULWA MUSILI**
PRINCIPAL INVESTIGATOR

THROUGH: **THE DIRECTOR, CVR,**
NAIROBI

DIRECTOR
CENTRE FOR VIRUS RESEARCH
P.O. BOX 54628
NAIROBI.

Dear Sir,

RE: KEMRI/SERU/CVR/002/3449 (RESUBMISSION OF *INITIAL SUBMISSION*): DETERMINATION OF VECTOR COMPETENCE OF *AEDES SIMPSONI* MOSQUITO COMPLEX FROM KILIFI (COASTAL) AND *AEDES VITTATUS* FROM WEST POKOT REGION FOR CHIKUNGUNYA VIRUS

Reference is made to your letter dated April 28, 2017. The KEMRI/Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on the May 5, 2017.

This is to inform you that the Committee notes that the issues raised during the 261st ERC meeting of the KEMRI/SERU held on **March 21, 2017** have been adequately addressed.

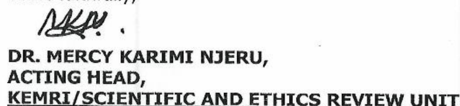
Consequently, the study is granted approval for implementation effective this day, **May 15, 2017** for a period of one year. Please note that authorization to conduct this study will automatically expire on **May 14, 2018.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **April 2, 2018**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received.

Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,



DR. MERCY KARIMI NJERU,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

In Search of Better Health

Appendix IV: Proposal Approval Letter From JKUAT -BPS

Forward to KEMRI

JOMO KENYATTA UNIVERSITY OF AGRICULTURE AND TECHNOLOGY
DEAN
30 AUG 2019
SIGN:
SCHOOL OF BIOMEDICAL SCIENCES

JOMO KENYATTA UNIVERSITY
OF
AGRICULTURE AND TECHNOLOGY
DIRECTOR, BOARD OF POSTGRADUATE STUDIES

P.O. BOX 62000
NAIROBI – 00200
KENYA
Email: director@bps.jkuat.ac.ke

TEL: 254-067-52711/52181(6114)
FAX: 254-067-52164/52030

REF JKU/2/11/ TM304-4136/2015

29th August 2019

FRANCIS MULWA
C/o SOBMS
JKUAT

Dear Mr. **Mulwa**

RE: APPROVAL OF RESEARCH PROPOSAL AND SUPERVISORS

Kindly note that your MSc. research proposal entitled "**Determination of vector competence of Aedes bromeliae population from kilifi and Aedes vittatus population from west pokot,Kenya for chikungunya virus**" has been approved. The following are your approved supervisors:-

1..Dr. Rosemary Sang

2. Dr. Joel Lutomiah

3.Dr. Michael Kahato

PROF. MATHEW KINYANJUI
DIRECTOR, BOARD OF POSTGRADUATE STUDIES

Copy to: Dean SOBMS

JKUAT is ISO 9001:2015 and ISO 14001:2015 Certified
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Appendix V: Plagiarism Report

Page 1 of 26

Plagiarism Detector v. 1092 - Originality Report:

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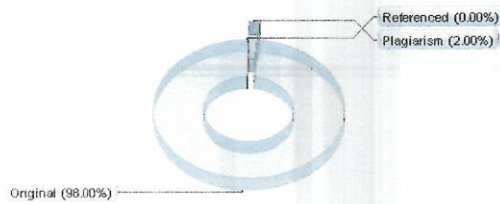

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Appendix VI: Published Paper

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Vector competence of *Aedes bromeliae* and *Aedes vitattus* mosquito populations from Kenya for chikungunya virus

Francis Mulwa[1]*, Joel Lutomiah[2], Edith Chepkorir[3], Samwel Okello[3], Fredrick Eyase[1,4], Caroline Tigoi[3,5], Michael Kahato[1], Rosemary Sang[2,3]

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OPEN ACCESS

Citation: Mulwa F, Lutomiah J, Chepkorir E, Okello S, Eyase F, Tigoi C, et al. (2018) Vector competence of *Aedes bromeliae* and *Aedes vitattus* mosquito populations from Kenya for chikungunya virus. PLoS Negl Trop Dis 12(10): e0006746. https://doi.org/10.1371/journal.pntd.0006746

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Data Availability Statement: All the relevant data are within the paper and its supporting information files.

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Background

Kenya has experienced outbreaks of chikungunya in the past years with the most recent outbreak occurring in Mandera in the northern region in May 2016 and in Mombasa in the coastal region from November 2017 to February 2018. Despite the outbreaks in Kenya, studies on vector competence have only been conducted on *Aedes aegypti*. However, the role played by other mosquito species in transmission and maintenance of the virus in endemic areas remains unclear. This study sought to determine the possible role of rural *Aedes bromeliae* and *Aedes vittatus* in the transmission of chikungunya virus, focusing on Kilifi and West Pokot regions of Kenya.

Methods

Four day old female mosquitoes were orally fed on chikungunya virus-infected blood at a dilution of 1:1 of the viral isolate and blood ($10^{6.4}$ plaque-forming units [PFU]/ml) using artificial membrane feeder (Hemotek system) for 45 minutes. The engorged mosquitoes were picked and incubated at 29–30°C ambient temperature and 70–80% humidity in the insectary. At days 5, 7 and 10 post-infection, the mosquitoes were carefully dissected to separate the legs and wings from the body and their proboscis individually inserted in the capillary tube containing minimum essential media (MEM) to collect salivary expectorate. The resultant homogenates and the salivary expectorates were tested by plaque assay to determine virus infection, dissemination and transmission potential of the mosquitoes.

Results

A total of 515 female mosquitoes (311 *Ae. bromeliae* and 204 *Ae. vittatus*) were exposed to the East/Central/South Africa (ECSA) lineage of chikungunya virus. *Aedes vittatus* showed high susceptibility to the virus ranging between 75–90% and moderate dissemination and

transmission rates ranging from 35–50%. *Aedes bromeliae* had moderate susceptibility ranging between 26–40% with moderate dissemination and transmission rates ranging from 27–55%.

Conclusion

This study demonstrates that both *Ae. vittatus* and *Ae. bromeliae* populations from West Pokot and Kilifi counties in Kenya are competent vectors of chikungunya virus. Based on these results, the two areas are at risk of virus transmission in the event of an outbreak. This study underscores the need to institute vector competence studies for populations of potential vector species as a means of evaluating risk of transmission of the emerging and re-emerging arboviruses in diverse regions of Kenya.

Author summary

Kenya experienced its first chikungunya outbreak in 2004/2005 along the coastal area, followed by sporadic outbreaks in Mandera in 2016, and subsequently in Mombasa city in late 2017 and early 2018. Despite the rising risk of transmission of the virus in the country based on evidence of outbreaks in Kenya, vector competence studies have only been limited to *Ae. aegypti*, while the role played by other *Aedes species* largely remain unknown. This study demonstrated the ability of *Ae. bromeliae* and *Ae. vittatus* to transmit chikungunya virus under controlled laboratory conditions. Vector competence remains the most important approach in disease risk assessment that provides knowledge to the public health sector in developing vector control guideline.

Introduction

Chikungunya virus (CHIKV) is vector-borne virus of genus *Alphavirus* and family *Togaviridae* that is principally transmitted from human to humans by *Ae. aegypti* and *Ae. albopictus*. The first CHIKV outbreak was documented in Makonde village in Tanzania in 1956 [1, 2] and since then, various outbreaks have been experienced in more than 60 countries in Africa, Asia, Europe and America [3, 4]. In Africa high infection was reported in union of Comoros island in the2004- 2005 outbreak [5], Congo in the 1998–2000 outbreaks [6] and Mauritius and Madagascar in 2005 and 2006 respectively [7]. CHIKV is re-emerging in Kenya, after the 2004–2005 outbreaks in Lamu Island. It has caused several outbreaks the northeastern and coastal Kenya from May 2016 and late 2017to early 2018 respectively [8]. In addition, previous studies have reported high seroprevalence rates (59%) of CHIKV infection in Busia District and 24% in Malindi Kenya [9].

Chikungunya virus strains are classified into three distinct genotypes; Asian, West African, and East/Central/South African (ECSA). This virus causes chikungunya fever, an acute febrile illness characterized by severe arthralgia, fever, skin rash, and arthritis-like pain in small peripheral joints that lasts for weeks or months, joint swelling and conjunctivitis [10–12]. Both *Ae. aegypti* and *Ae. albopictus* have been implicated in the CHIKV transmission cycle in the African region and other parts of the world, based on vector competence studies [13, 14] and virus isolation from infected field collected mosquitoes [15–17]. International travels and global expansion leading to the spread of the two main CHIKV urban mosquito vectors, Ae.

aegypti and *Ae. albopictus*, have enhanced the ability of the virus to spread to new regions where environmental conditions are permissive for viral transmission [18–20]. Extrinsic incubation period (EIP) in mosquitoes infected with CHIKV ranges from 2 to 9 days, with an average of 3 days in the tropics such as East Africa [21]

Aedes simpsoni consists of a complex of mosquito species including vectors of important arbovirus diseases such as yellow fever. In Kenya, *Ae. bromeliae* is the dominant species of the *Ae. simpsoni* complex found in the peridomestic areas, *Ae simponi simpsoni* has never been documented in the country [22]. Studies involving the ecology and vector competence of *Ae. vittatus* and *Ae. bromeliae* on chikungunya have been conducted in Senegal [23], and on dengue, and yellow fever virus in Kenya [24]. In Rabai, Kenya, *Ae. bromeliae* breeds in the domestic and peridomestic areas while *Ae. lilii* breeds in the forest [25, 26]. *Aedes bromeliae* preferably feed on human hosts for their blood meal, maintaining the virus in the rural cycle [24] and breed not only on water reservoirs held by plants, including trees holes and plant leaf axils [27, 28], but also in artificial water containers [29, 30].

Aedes vittatus is a savannah species that is abundant in rocky areas, prevalent in African forest galleries and is also common in villages near forests. Female *Ae. vittatus* have daily and nocturnal activities with a significant crepuscular peak [23, 31]. They bite a wide range of vertebrate hosts, with a strong anthropophilic trend in specific locations [32], and breed mostly on natural habitats mainly in rock pools/holes and tree holes during the rain seasons. In absence of these breeding sites the vector breeds in domestic areas especially in household water-holding containers [23]. The vector has a high susceptibility to infection and dissemination, and most importantly is able to transmit the West Africa lineage of CHIKV [23, 33]. *Aedes vittatus* and *Ae. bromeliae* have the potential to expand their distribution and abundance due to their ability to adapt to human dwellings using available breeding habitats, such as domestic containers, in absence of their preferred breeding sites [26, 33, 34].

Determination of the vector competence of mosquito populations is a key parameter in evaluating the risk of CHIKV transmission and spread in Kenya. Despite several outbreaks of CHIKV in Kenya, focus is usually on *Ae. aegypti* and no vector competence studies have been conducted to determine the role played by other mosquito species in its transmission and maintenance. We evaluated the competence of *Ae. bromeliae* populations from Rabai subcounty in Kilifi County and *Ae. vittatus* populations from Kacheliba sub-county in West Pokot County of Kenya as an important factor in assessing the risk of transmission of ECSA lineage of CHIKV in these regions. This would provide the necessary baseline data to inform the public health sector on best vector control practice, and effective preventive and control interventions in case of increased risk of virus transmission.

Materials and methods

Study sites

This study was conducted in Rabai sub-county, Kilifi County in the coastal region of Kenya and Kacheliba sub-county in West Pokot County (Fig 1). Kilifi County (latitude 3.63°S, longitude 39.85°E) has a mean annual temperature of 30°C, relative humidity of 82% and receives approximately 88.25 mm of rainfall annually. The county has a bimodal pattern of rainfall with the long rains occurring between April and July, with the highest rainfall occurring in the month of May and short rains in November and December. In Kilifi, the rainfall patterns towards the hinterlands are unreliable due to the influence of the Indian Ocean. The main topographical features include the coastal plains, island plains and Dodori River Plain. The presence of forest areas around the town inhabited by primates and other wildlife species poses a risk of zoonotic disease transmission. Minimum temperatures are always above 20°C,

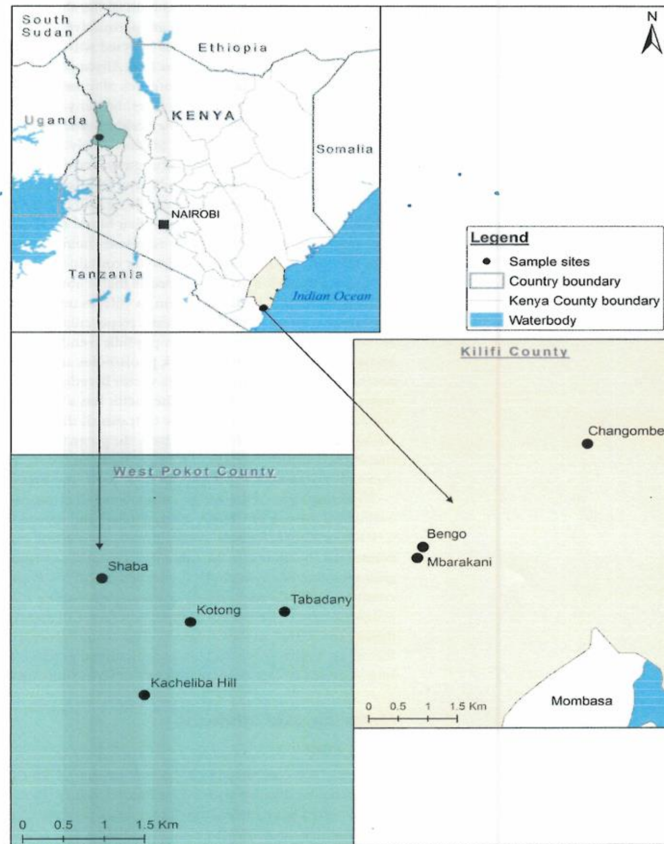

Fig 1. Map of Kenya showing the study sites.

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the maximum temperatures reach 30°C to 34°C. The natural vegetation consists of coconut trees, banana plantations and a variety of agricultural crops. Characteristic soil types consist of sandy soil with patches of high loam soil.

West Pokot County lies between latitudes 1.13°N to 2.70°N and longitudes 34.77°E to 35.79°E in the Rift Valley region of Kenya, bordering the Republic of Uganda to the west, Trans-Nzoia County to the south, Elgeyo-Marakwet and Baringo Countiesto the southeast and Turkana County to the north and northeast. It covers an area of 9,169.39 km^2. West Pokot County has a bimodal rainfall pattern. The long rain season occurs between May and June with mean daily temperature of 32°C, rainfall of approximately 60.25 mm and 82% relative humidity.

Mosquito collection

Aedes bromeliae eggs, larvae and pupae were collected from peridomestic areas in four villages in Rabai sub-county: Mbarakani, Bengo, Changombe and Kibarani (Fig 1). The eggs were collected using ovitraps that consisted of black ovicups lined with oviposition paper and half-filled with water. After obtaining consent from the home/residence owner to sample in their private land, the ovitraps were placed in the peridomestic areas for four days to allow the mosquitoes to lay eggs. Larvae and pupae were collected from natural habitats, mainly rock pools/holes and tree holes, plant axils, especially bananas, and flower axils using larval sampling tools. *Aedes vittatus* larvae and pupae were collected from rock pools/holes and tree holes in peridomestic and forest areas of Kacheliba sub-county. Field collected eggs were briefly dried on a damp cotton wool to induce diapause, and transported to a level 2 (BSL2) insectary at Kenya Medical Research Institute (KEMRI) for colonization.

Mosquito rearing and identification

To avoid an oviposition from a single female mosquito, several larval collections from the same area were mixed. All collected larvae and pupae were reared to adults in the field laboratory and then transported to the KEMRI insectary for identification. In the insectary, the oviposition papers with eggs were dispensed in water to allow hatching and the emerging larvae were fed on fish fingerlet meal (Tetramin baby) until pupation. The pupae were transferred in small cups containing water to within 4 liter plastic cages with netting material for eventual development to adults. The adults were knocked down at -20°C for 45 seconds and morphologically identified using an identification key [35–38] under a dissecting microscope to select *Ae. bromeliae* and *Ae. vittatus* for use in the study. The adult mosquitoes were provided with 10% glucose solution on cotton wool and maintained at temperature between 28–32°C, 70–80% relative humidity and 12:12 hour light:dark (L:D) photoperiod. In order to stimulate egg production the mosquitoes were fed on anaesthetized clean laboratory mice placed on top of the cage for 45 minutes. The eggs collected were hatched into F_1 (first filial generation) and adult mosquitoes were maintained as described.

Virus and virus amplification

The Lamu001 strain of ECSA lineage CHIKV, isolated from human during the 2004–2005 outbreak in Lamu Island [6], was used for all the infection assays performed in this study. The virus was passaged in confluent monolayers of Vero cells in T-25 cell culture flasks, grown in Minimum Essential Medium (MEM), (Sigma-Aldrich, St. Louis, MO) with Earle's salts and reduced $NaHCO_3$, supplemented with 10% heat inactivated fetal bovine serum **Fetal bovine serum** (or foetal bovine serum) is serum taken from the fetuses of cows. Fetal Bovine Serum (or FBS) is the most widely used serum in the culturing of cells. In some papers the expression

foetal calf serum is used. (FBS FBS *abbr.* fasting blood sugar FBS Fasting blood sugar. See Fasting glucose.), (Sigma-Aldrich), 2% L-glutamine (Sigma-Aldrich) glutamine (gl'təmēn), organic compound, one of the 20 amino acids commonly found in animal proteins. and 2% antibiotic antimycotic solution containing 10,000 units penicillin, 10 mg streptomycin and 25μg amphotericin B per ml (Sigma-Aldrich, St. Louis, MO). The inoculated monolayer was incubated at 37°C for 1 hour, to allow for virus adsorption and then maintenance medium (MEM, with 2% Fetal Bovine Serum, 2% glutamine, 2% antibiotic/antimycotic) was added and incubated at 37°C. 80% cytopathic effect (CPE) was observed after two days. The **CPE**—Customer Premises Equvirus was harvested, aliquoted in cryovials and stored at -80°C until use [39].

Virus quantification

Quantification of CHIKV was performed by plaque assay. 10-fold serial dilutions of the amplified CHIKV was carried out and inoculated in 6-well plates containing confluent Vero monolayers as described by Gargan [40]. This was 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, 10 mg streptomycin and 25 μg amphotericin B per ml and incubated at 37°C in 5% CO_2 overnight. Each well was inoculated with 100 μl of the respective virus dilution, incubated for 1 hour with frequent rocking to allow for adsorption. The infected cells were maintained using 2.5% methylcellulose mixed with 2X maintenance medium (MEM, GIBCO Invitrogen corporation, Carlsbad, California) and incubated at 37°C with 5% CO_2 for 4 days; then fixed for 1 hour with 10% formalin, stained for 2 hours with 0.5% crystal violet, washed and the plaques counted and calculated to quantify the virus using the following formula [39]:

$$\frac{Number\ of\ plaques}{d \times V} = PFU/ml$$

where d is the dilution factor and V is the volume of diluted virus added to the wells.

Oral infection of mosquitoes

The wild filial generation (F_0) and first generation (F_1) of female *Ae. bromeliae* and *Ae. vittatus*, respectively, were deprived of glucose for 24 hours before exposure to the infectious blood meal, using an artificial membrane feeding system (Hemotek). The virus/blood mixture was put in membrane feeders covered with freshly prepared mouse skin, and maintained using the hemotek system which employs an electric heating element that maintains the temperature of the blood meal at 37°C. Batches of 50–100 female mosquitoes aged 4–5 days were fed on the virus-blood mixture at a ratio of 1:1 (CHIKV isolate and defibrinated sheep blood) using a Hemotek feeding system for 60 minutes. Only fully engorged mosquitoes were transferred to 4-litre plastic cages (15–30 mosquitoes/cage) with a net on top and maintained with 10% glucose at 28–30°C, relative humidity of 70–80%, and 12:12 hour L: D photoperiod. The non-engorged mosquitoes were destroyed. Mosquito mortality was monitored in the cages by removing and counting dead mosquitoes daily. The experiment was done in three replicates to obtain the sufficient sample size.

Infection and dissemination rate

On 5, 7 and 10 days post-infection (dpi), a representative sample (at least 30%) of the orally exposed mosquitoes were picked, cold anesthetized and carefully decapitated with the legs/

wings and bodies placed into separate 1.5 mL microfuge tubes (Eppendorf). Each mosquito body was placed separately in a well labelled 1.5ml tubes containing 1000 μl of homogenization media (HM), made of MEM, supplemented with 15% FBS, 2% L-glutamine, and 2% antibiotic/antimycotic. Mosquito bodies were homogenized using a mini bead beater (BioSpec Products Inc, Bartlesville, OK 74005 USA) with the aid of a copper bead (BB-caliber airgun shot) and clarified by centrifugation at 12,000 rpm (Eppendorf centrifuge 5417R) for 10 minutes at 4°C. The supernatants were inoculated in Vero cells in 12 well plates, grown in MEM, supplemented with 10% FBS, 2% L-glutamine and 2% antibiotic/antimycotic. One hundred microliters of the appropriate dilutions of the abdominal homogenates was added to each of ten wells of the 12-well plate to infect the cells and the remaining two wells were used for controls, negative control was comprised of male mosquitoes from the study vectors comprising of a pool of 25 mosquitoes. The plates were incubated at 37°C in a 5% CO_2 incubator with frequent agitation after every 15 minutes for 1 hour to allow for virus adsorption. The infected cell monolayers were then overlaid with 2.5% methylcellulose supplemented with 2% FBS, 2% L-Glutamine and 2% antibiotic/antimycotic and incubated at 37°C in 5% CO_2. On day 4, plates were fixed for 1 hour with 10% formalin, and stained for 2 hours with 0.5% crystal violet, washed on running tap water, dried overnight and the plaques observed on a light box. The CHIKV positive bodies were used to determine the infection rates. For each positive abdomen, corresponding legs were homogenized and their infection status determined as described above for the abdomens. Plaques were counted and calculated to determine the viral titer. If the virus was detected in the mosquito's body but not in the legs, the mosquito was considered to have a non-disseminated infection, limited to the midgut. Detection of virus in the body and legs was considered evidence of successful infection and dissemination, respectively [41].

Test for transmission potential

After exposing the mosquitoes to the infectious blood meal, engorged mosquitoes were picked, placed into new cages, reared under the insectary conditions and maintained with 10% sucrose. On 5, 7 and 10 days dpi, mosquitoes were sucrose-starved and deprived of water for 16 hours, then cold anesthetized for about 40 seconds before the legs and wings from each of them were carefully removed and placed on sticky tape. Individual mosquito proboscises were inserted into a capillary tube containing 10–20 ul HM. Mosquitoes were allowed to expectorate saliva for 30 minutes. Media containing saliva was then expelled into a cryovial containing 200 ul of MEM and stored at -80°C until tested. A volume of 80 μl of the saliva sample was inoculated into each well of a 24-well plate containing confluent Vero cell monolayers. Plates were incubated for 1 hour to allow for adsorption, with frequent agitation. The infected cells were maintained using maintenance media (1 ml per well) and incubated at 37°C with 5% CO_2. Plates were observed for 7 days and the supernatant of wells showing CPE were harvested and virus quantified by plaque assay as discribed above. Plaques were counted and calculated to quantify the virus.

Ethical considerations

Scientific and ethical approval to carry out this study was obtained from the KEMRI Scientific Ethical Review Unit (SERU) (KEMRI/SERU/CVR/002/3449). The animal use component was reviewed and approved by KEMRI Animal Care and Use Committee (ACUC) (KEMRI/ACUC/01.05.17). The KEMRI ACUC adheres to national guidelines on the care and use of animals in research and education in Kenya enforced by National Commission for Science, Technology and Innovation (NACOSTI). The Institute has a foreign assurance identification number F16-00211 (A5879-01) from the Office of Laboratory Animal Welfare (OLAW) under

the Public Health Service and commits to the International Guiding Principles for Biomedical Research Involving Animals.

Data and statistical analysis

Three parameters describing vector competence were determined: infection (number of infected mosquito bodies per 100 mosquitoes orally exposed and tested), dissemination (number of mosquitoes with positive legs per 100 mosquitoes infected) and transmission rates (number of mosquitoes with positive saliva per 100 mosquitoes with disseminated infection).

Test of proportions were used to get the infection, transmission and dissemination rates with their 95% confidence interval (CI). Chi-square test with or without Yates' correction or Fisher's exact test were used to assess the differences between the two species at each time point and between the three time points for each species. Test of difference between means was done for the titers to determine if there was significant difference in incubation days for each species for the infection and dissemination. Statistical significance was considered for $p < 0.05$.

Results

Over 90% of all the larvae and pupae that were collected from plant leaf axils were *Ae. bromeliae* while over 70% of larvae and pupae that were collected from rock pools and tree holes were *Ae. vittatus*. The mosquito species used in this study and the breeding habitats where they were collected are presented below (Table 1).

Aedes bromeliae and *Ae. vittatus* infection, dissemination and transmission potential

The feeding success rate of the two mosquito species on infected blood meal was high, ranging from 70–80% for *Ae. bromeliae* and 40–50% for *Ae. vittatus*. The blood meal titres were determined before and immediately after mosquito exposure. The infection rate for Kilifi and West Pokot mosquitoes were measured from a total of 311 *Ae. bromeliae* (110 on 5 dpi, 101 on 7 dpi and 100 on 10 dpi) and 204 *Ae. vittatus* (69 on 5 dpi, 69 on 7 dpi and 66 on 10 dpi). Both species were susceptible to chikungunya virus infection with average infection rates of 37% and 79% for *Ae. bromeliae* and *Ae. vittatus*, respectively. *Aedes vittatus* had high midgut infection rate, with no significant difference between the extrinsic incubation periods. The overall dissemination rate was high for *Ae. vittatus* with more than 46% of the mosquitoes with midgut infection having a disseminated infection. *Aedes bromeliae* had moderate midgut infection rate on 5 and 7 dpi, but low infection rate on 10 dpi. Overall *Ae. bromeliae* showed relatively low dissemination with 34% of those with midgut infection having disseminated infection (Table 2).

Table 1. Mosquito species and their preferred breeding habitats.

Mosquito species	Habitat	Breeding sites	Date of collection	Stage
Ae. bromeliae	Peridomestic	Banana leaf axils	Jul-2017	larvae, pupae
		Arrow root leaf axils	Jul-2017	larvae, pupae
		Flower axils	Jul-2017	larvae, pupae
Ae. vittatus	Peridomestic	Rock pools/holes	May-2017	Eggs, larvae, pupae
	Forest	Rock pools/holes	May-2017	Eggs, larvae, pupae
		Tree holes	May-2017	Eggs, larvae, pupae

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Table 2. Infection, dissemination and transmission rates of mosquitoes orally infected with CHIKV (infectious blood meal = $10^{6.4}$ PFU/mL).

		Day 5		Day 7		Day 10	
		n	Infection rate% (95%CI)	n	Infection rate% (95%CI)	n	Infection rate% (95%CI)
Percentage of infection	*Ae. bromeliae*	45	40.9 (31.6–50.7)	44	43.6 (33.7–53.8)	26	26.0 (17.7–35.7)
	Ae. vittatus	56	81.2 (69.9–89.6)	54	78.3 (66.7–87.3)	52	78.8 (67.0–87.9)
	p value		**<0.001**		**<0.001**		**<0.001**
Percentage of disemination	*Ae. bromeliae*	12	26.7 (14.6–41.9)	16	36.4 (22.4–52.2)	11	42.3 (23.4–63.1)
	Ae. vittatus	26	46.4 (33.0–60.3)	23	42.6 (29.2–56.8)	26	50.0 (35.8–64.2)
	p value		**0.042**		0.531		0.521
Percentage of transmission	*Ae. bromeliae*	5	41.7 (15.2–72.3)	5	31.3 (11.0–58.7)	6	54.5 (23.4–83.3)
	Ae. vittatus	11	42.3 (23.4–63.1)	11	47.8 (26.8–69.4)	9	34.6 (17.2–55.7)
	p value		0.97		0.301		0.259

https://doi.org/10.1371/journal.pntd.0006746.t002

Aedes bromeliae and *Ae. vittatus* susceptibility to CHIKV infection, dissemination and transmission

Aedes vittatus was highly susceptible to CHIKV with infection rates of 81%, 78%, and 79% on 5, 7 and 10 dpi respectively compared to *Ae. bromeliae* which was moderately susceptible with infection rates of 44%, 41% and 26% on 5, 7 and 10 dpi respectively (Fig 2A). Infection rates for *Ae. vittatus* were higher relative to that of *Ae. bromeliae*. Statistically significant differences were observed for infection rates 5 dpi between *Ae. bromeliae* (40.9%, 95% CI [31.6–50.7%]) and *Ae. vittatus* (81.2%, 95% CI [69.9–89.6%]) $p < 0.001$; 7 dpi between *Ae. bromeliae* (43.6%, 95% CI [33.7–53.8%]) and *Ae. vittatus* (78.3%, 95% CI [66.7–87.3%]) $p < 0.001$; and 10 dpi between *Ae. bromeliae* (26.0%, 95% CI [17.7–35.7%]) and *Ae. vittatus* (78.8%, 95% CI [67.0–87.9%]) $p < 0.001$ (Table 2). Dissemination rates for *Ae. vittatus* were higher relative to those of *Ae. bromeliae*. However, statistical significant difference was only observed 5 dpi, *Ae. bromeliae* (26.7%, 95% CI [14.6–41.9%]) and *Ae. vittatus* (46.4%, 95% CI [33.0–60.3%]) $p < 0.042$. Viral dissemination was observed as early as 5–7 dpi for both species. The proportion of disseminated infection for *Ae. bromeliae* increased significantly with increase in the number of days post infection with higher rate on day 10 (43%). *Aedes bromeliae* had dissemination rate of 26%, 36% and 43% at 5, 7 and 10 dpi (Fig 2B). *Aedes vittatus* had disseminated infection rates of 46%, 43% and 50% at 5,7 and 10 dpi, respectively, but these differences were not statistically significant (chi-square test, $p>0.05$) (Fig 2B). The overall data shows that 114 out of 277 mosquitos with midgut infection disseminated the virus to the legs, the *Ae. vittatus* population from West Pokot County had higher dissemination rate (46%), than the *Ae. bromeliae* (34%) population from Kilifi county. Both species were able to transmit the virus as early as 5 dpi. The transmission rate for *Ae. bromeliae* was higher on day 10 (55%) compared to other days post infection. *Aedes vittatus* had higher

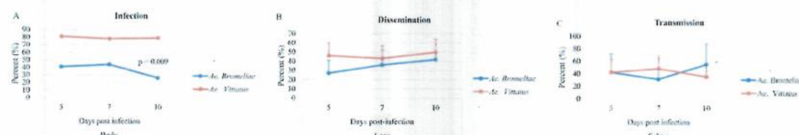

Fig 2. Proportion of Kilifi, *Ae. bromeliae* and West Pokot, *Ae. vittatus* infected with CHIKV at 5, 7 and 10 days post infection, infection rate (A) and dissemination rate (B) and transmission rate (C).

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transmission rate on day 7 (48%) which significantly declined on day 10 (35%) post infection (Fig 2C). The overall data for both the Kilifi and West Pokot mosquito population shows that 46 out of 114 (40%) were able to transmit the virus. Although *Ae. vittatus* had higher infection and dissemination, there was no significant difference on overall transmission in both vectors (*Ae. vittatus* 41% and *Ae. bromeliae* 41%).

Aedes bromeliae dissemination efficiencies increased with increase in the number of days post infection, *Ae. vittatus* had high dissemination efficiencies on 7 dpi (Table 2). Overall transmission rates for *Ae. vittatus* was higher (Fig 2) relative to that of *Ae. bromeliae* though no statistical significance was observed (chi-square test, $p>0.05$).

Chikungunya virus replication dynamics in the analyzed mosquito populations

Aedes bromeliae and *Ae. vittatus* were analysed to assess viral titers in bodies and legs plus wings by titration in Vero cells. *Aedes bromeliae* bodies showed mean viral titers of 5.0 ± 0.33 $\log_{10}$ PFU/mL, 5.3 ± 0.34 $\log_{10}$ PFU/mL, 5.3 ± 0.45 $\log_{10}$ PFU/mL at 5, 7, and 10 days post infection, respectivelyn. The mean CHIKV titers in the bodies increased progressively, reaching a value of 5.3 ± 0.45 $\log_{10}$ PFU/mL 10 dpi (Fig 3). The viral presence in the legs was detected as early as 5 dpi with a titer of 4.0 ± 0.58 $\log_{10}$ PFU/mL, and titers of 4.3 ± 0.52 $\log_{10}$ PFU/mL and 4.3 ± 0.62 $\log_{10}$ PFU/mL on day 7 and 10 dpi, respectively.

Aedes vittatus bodies showed mean viral titers of 5.7 ± 0.32 $\log_{10}$ PFU/mL, 5.8 ± 0.32 $\log_{10}$ PFU/mL, 4.9 ± 0.31 $\log_{10}$ PFU/mL at 5, 7, and 10 dpi, respectively (Fig 3). The viral presence in the legs was detected as early as 5 dpi with a titer of 3.6 ± 0.37 $\log_{10}$ PFU/mL, and titers of

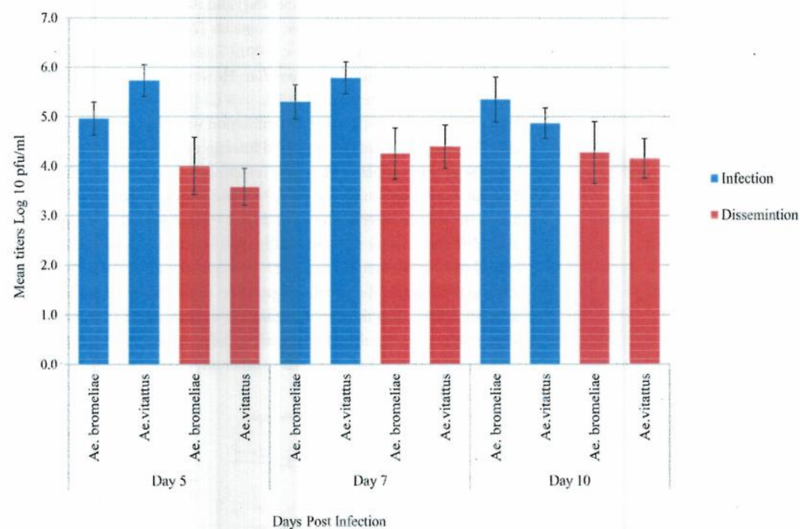

Fig 3. Chikungunya virus replication in *Ae. bromeliae* and *Ae. vittatus*. Comparisons of CHIKV mean titer in infected *Ae. bromeliae* and *Ae. vittatus* females was calculated by titration on VERO cells, samples were collected at different days post infection and individually analysed for the presence of CHIKV in body and legs plus wings.

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Table 3. Mean body and leg titers for *Ae. vittatus* and *Ae. bromeliae* exposed to chikungunya virus.

species	Non disseminated	Disseminated [b]		Mean leg titer [c]	
	Body titers [a]	Body titer	Leg titer	No transmission	Transmission
Ae. bromeliae	$10^{4.9}$	$10^{5.8}$	$10^{4.1}$	10^{4}	$10^{4.3}$
Ae. vittatus	$10^{5.4}$	$10^{5.9}$	10^{4}	$10^{3.9}$	$10^{4.4}$

[a] Mean body titer for infected mosquitoes with negative legs (PFU/specimen)

[b] Mean titers for infected mosquitoes with positive legs (PFU/specimen)

[c] Mean leg titers for virus-positive legs with negative saliva (No transmission) and those with positive saliva (Transmission) (PFU/specimen).

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4.4 ± 0.44 $\log_{10}$ PFU/mL and 4.2 ± 0.40 $\log_{10}$ PFU/mL 7 and 10 dpi respectively. Our results highlighted that among our *Ae. bromeliae* and *Ae. vittatus* populations, CHIKV was able to infect mosquitoes and replicate over time, disseminating to the wings and legs and reaching the salivary glands. There was no significant difference in infection and dissemination mean titers between the vectors.

In general, viral dissemination only occurred when body titers were $\geq 10^5$ for both strains. *Ae. bromeliae* had a midgut infection barrier that was stronger than that of *Ae. vittatus*. No difference in leg titers was observed between mosquitoes that did and did not transmit the virus (Table 3). No statistical difference for mean titers for the *Ae. bromeliae* and *Ae. vittatus* observed for all timepoints (chi-square test, $p>0.05$).

Discussion

This is the first study to determine the ability of *Ae. bromeliae* and *Ae. vittatus* mosquito populations from Kenya to transmit the ECSA lineage of CHIKV. This study has demonstrated that the two are laboratory competent vectors for ECSA lineage of CHIKV. The recent outbreak of chikungunya in Africa, America, Asia and Europe [18, 42, 43], clearly demonstrates the potential of the disease to spread to new areas and cause massive epidemics. The risk of importation of CHIKV to new areas is due to international and local travels from epidemic areas and exporting infected vectors to new areas where there are susceptible people and competent vectors [14, 44]. The full competence of a vector is not only determined by the ability of the vector to get infected, but also by its ability to transmit the pathogen [45]. In this study we determined the capacity of the vectors to get infected, disseminate and transmit the virus.

The CHIKV titers ($10^{6.4}$ PFU/ml) used to infect mosquitoes in this study, are similar to published viremia levels associated with human infections (often $>10^5$ PFU/mL blood) in nature [46]. It has also been shown that a titer of 10^4 PFU/ml in monkeys was sufficient to infect mosquitoes [41]. Our results show that these two mosquito species are susceptible to infection and have ability to transmit CHIKV (Table 2). Although all mosquito species tested had ingested infectious blood meals, not all mosquitoes were infected and not all that were infected had the virus disseminated. This shows that other factors, such as the midgut escape barrier, affect the replication and dissemination of the virus in a mosquito [47].

The *Ae. bromeliae* population had moderate midgut infection which ranged from 26–44% across the different days post infection. Virus infection in the midgut was detected as early as 5 dpi. This is similar to previous studies which showed that the mosquito bodies infection with CHIKV in East Africa ranges from 2–9 days [21]. *Aedes bromeliae* had the highest transmission rate 10 dpi, compared to *Ae. vittatus*, which had its highest transmission rate 7 dpi, suggesting *Ae. bromeliae* requires more days for the virus to infect the salivary glands and eventually transmit to a susceptible host. Aedes vittatus breeds mostly on rock pools/holes and tree holes as demonstrated by their representing over 70% of the total collected in these

habitats. Breeding of Ae. vittatus in rock pools and tree holes has been previously documented [23, 33, 48]. This study showed that the West Pokot population of Ae. vittatus has the potential to transmit CHIKV as has been demonstrated in other studies [23]. Our data showed that Ae. vittatus midgut infection and dissemination rates 5 dpi were relatively high suggesting the presence of weak midgut infection and escape barriers. Our data suggest the West Pokot Ae. vittatus population is efficient in transmitting CHIKV and indicates a potential risk if the virus is introduced in the area.

Our study demonstrated that not all *Ae. bromeliae* and *Ae. vitattus* are capable of transmitting the CHIKV via capillary feeding; showing that dissemination is dependent on the midgut infection [49]. However, such *in vitro* experiments may not represent the actual amount of virus inoculated in a host during feeding. Despite the two species being exposed to the same virus titers, *Ae. vittatus* showed high infection and dissemination rates compared to *Ae. bromeliae*. This may be due to other intrinsic factors such as varying strength of midgut infection barrier and midgut escape barrier that individually affect the susceptibility of different mosquito species to infections [50]. It was observed that *Ae. vittatus* had a higher midgut infection than *Ae. bromeliae*, but there was no significant difference in transmission between the two species regardless of the incubation period. Since this is determined by the ability of the virus to penetrate into the saliva glands and be secreted into the saliva, the data support the notion that the salivary gland barrier is independent of the midgut infection and [51]. For both species, a higher viremia in their infected legs correlated with the ability to transmit the virus by the capillary method. Although this method is not a fully accurate representation of transmission, it does confirm the presence of virus in the salivay and can be used as a model to test for transmission of viruses which have no documented animal models for such experiments. Mosquitoes usually secrete less virus into a capillary tube than when feeding on an animal [52] and transmission rates are often lower when they are determined by collection of saliva as compared to allowing the mosquito to feed naturally on a susceptible animal [53]. Therefore, failure to detect CHIKV in the saliva collected in a capillary tube does not necessarily mean that the mosquito would not have transmitted the virus by bite if it fed on a susceptible human. In this case, our transmission rates should be considered as minimum transmission rates. Additionally, although *Ae. vittatus* and *Ae. bromeliae* from Kenya are efficient laboratory vectors, their potential role in CHIKV transmission depends on other factors in relation to mosquito ecology such as densities, survival, longevity, anthropophily and duration of gonotrophic cycles, which have been shown to interfere with transmission and maintenance of CHIKV.

Conclusion

This study demonstrated that *Ae. vittatus* and *Ae. bromeliae* populations in Kenya are laboratory competent vectors of ECSA lineage CHIKV and it indicates the potential for CHIKV transmission to occur in these locations should the virus find its way there through travel or introduction via a sylvatic host. It is therefore recommended that the public health authorities should continually monitor and carry out surveillance of the CHIKV and virus genotypes circulating within particular regions as well as identify vectors mediating these transmissions to prevent their adverse effects before an outbreak.

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Methodology: Francis Mulwa, Joel Lutomiah, Edith Chepkorir, Samwel Okello, Michael Kahato, Rosemary Sang.

Project administration: Francis Mulwa, Edith Chepkorir, Caroline Tigoi.

Resources: Rosemary Sang.

Supervision: Francis Mulwa, Joel Lutomiah, Samwel Okello, Fredrick Eyase, Caroline Tigoi, Michael Kahato, Rosemary Sang.

Validation: Francis Mulwa.

Visualization: Francis Mulwa, Rosemary Sang.

Writing – original draft: Francis Mulwa.

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