THE EFFECT OF REARING TEMPERATURE ON THE VECTOR COMPETENCE OF *AE. AEGYPTI* MOSQUITO POPULATIONS FROM KILIFI AND NAIROBI COUNTIES FOR DENGUE-2 VIRUS

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The Effect of Rearing Temperature on the Vector Competence of *Ae. aegypti* Mosquito Populations from Kilifi and Nairobi Counties for Dengue-2 Virus

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DECLARATION

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

Sign……. Date…….....

Edith Chepkorir

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

Sign…… Date……

Prof. Zipporah Ng'ang'a

JKUAT, Kenya

Sign…… Date……

Dr. Rosemary Sang

KEMRI), Kenya

DEDICATION

This thesis is dedicated to my father and mother, who taught me that even the largest task can be accomplished if it is done one step at a time.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURE

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMNS

ACUC	Animal care and use committee
AVID	Arbovirus Incidences and Diversity
BSL	Biosafety level
CI	Confidence Interval
CPE	Cytopathic effect
CVR	Center for Virus Research
DENV	Dengue virus
DMEM	Dulbecco's modified eagles media
DNA	Deoxyribonucleic acid
ERC	Ethical review committee
FBS	Fetal bovine serum
GPS	Global positioning system
HM	Homogenization medium
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
MEM	Minimum essential medium
MEB	Midgut escape barrier
Mg	Miligrams
MIB	Midgut infection barrier
Ml	Mililitres
Pfu	Plaque forming units
PPE	Personal protective equipment
RH	Relative humidity
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase – Polymerase chain reaction
SSC	Scientific steering committee
TOT	Transovarial transmission
VT	Vertical transmission
WHO	World Health Organization

ABSTRACT

Susceptibility of *Ae. aegypti* mosquito to dengue virus (DENV) varies geographically and can be influenced by climatic factors such as temperature, which affect the incidence, seasonality and distribution of vector-borne diseases. The first outbreak of dengue fever (DF) in Kenya occured in 1982 in the coastal towns of Malindi and Kilifi. Unlike Nairobi where no active dengue transmission has been reported, DF is currently re-emerging at the Coast causing major outbreaks. This study investigated the effect of rearing temperature on the vector competence of *Ae. aegypti* populations from Kilifi and Nairobi counties for dengue-2 virus. A total of 1,117 four-day old adult female *Ae. aegypti* mosquitoes collected as eggs from the two sites were artificially exposed to defibrinated sheep blood mixed with dengue-2 virus ($10^{5.08}$ PFU/ml) using a membrane feeder. Half of the exposed mosquitoes were incubated at high temperature (30°C) and the other half at low temperature (26°C), and every 7 days up to day 21 post-infection 30% of the exposed mosquitoes were randomly picked, individually dissected, separated into abdomen and legs, and tested for midgut and disseminated infection, respectively, including virus quantification by plaque assay using Vero cells. Nairobi mosquito population exhibited a significantly higher midgut infection rates (16.8%) compared to the Kilifi population (9%; p=0.0001). Midgut infection rates among the populations varied with temperature, with a significantly higher infection rate observed in mosquitoes from Nairobi at high (21.3%) compared to low temperature (12.0%; P=0.0037). Similarly, for Kilifi mosquito population, a significantly higher infection rate was recorded at high (11.6%) relative to low temperature (6.8%; P=0.0162). Disseminated infection was higher among the Kilifi mosquito population (40.7%) than in mosquitoes from Nairobi (10.3%; P<0.0001). The findings show that *Aedes aegypti* mosquito populations from Nairobi and Kilifi are susceptible to dengue-2 virus, with only Kilifi *Ae. aegypti* population supporting disseminated infection. It was observed that infected *Ae. aegypti* mosquitoes are able to vertically transmit the virus to their progeny. This is the likely mechanism by which the virus is maintained and propagated among its natural hosts.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Dengue virus, a mosquito-borne virus belonging to the genus *Flavivirus* and family *Flaviviridae,* exists in 4 distinct serotypes (DENV 1-4) (Gubler & Clark, 1995). The virus is transmitted by *Aedes aegypti,* a day-biting domestic mosquito that breeds in natural or artificial water (Gubler, 2002). Dengue virus constitutes a major public health concern, causing more illness and death than any other arbovirus disease of humans. Each year, an estimated 100 million cases of dengue fever and several hundred thousand cases of Dengue Haemorrhagic Fever occur, depending on epidemic activity globaly in tropical and subtropical areas (Gubler, 1998). The World Health Organization estimates that 3.5 billion people in 128 countries are at risk of infection with dengue viruses (WHO, 2009). Dengue illness in humans presents with a wide spectrum of clinical manifestations, ranging from a flu-like Dengue Fever (DF), to the more severe Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS).

The first reported epidemics of dengue fever occurred in 1779-1780 in Asia, Africa, and North America (Gubler, 1998). Since then, dengue fever caused by all four serotypes has been on the increase since 1980, mostly affecting Asia, South America and the Caribbeans (Jansen &Beebe, 2010; Amarasinghe *et al.,* 2011). Although there is limited documentation on the burden of Dengue in Africa (Amarasinghe *et al.,* 2011) recent events suggest major re-emergence of the disease in Africa with outbreaks having been reported in East Africa (South Sudan, Somalia, Kenya and Tanzania) (AFEN, 2013) and West Africa (Senegal, Central Africa Republic) (Faye *et al.,* 2014). In Kenya, the first outbreak of dengue fever caused by dengue-2 virus (DENV-2) occured in 1982 in the coastal towns of Malindi and Kilifi (Johnson *et al.,* 1982). A serosurvey carried out in 2005 revealed the occurrence of dengue transmission in coastal and inland parts of Kenya (Mease *et al.,* 2011). In 2012, dengue outbreak was reported in Northern Kenya and subsequently in the coastal

town of Mombasa (AFEN, 2013; KEMRI, 2012-2014) and is now viewed as a re-emerging public health problem in Kenya.

Dengue virus is transmitted by female mosquitoes mainly of the species *Aedes aegypti* and, to a lesser extent, *A. albopictus*. *Aedes aegypti* mosquito, the principal vector of DENV, originated in Africa and spread to tropical countries in the 17th and 18th Centuries (Halstead, 2008). Urbanization is a major factor in facilitating the increase of *Aedes* mosquito species populations (Gubler & Clark, 1995). Accumulation of non-biodegradable, man-made containers used to store water in and around living areas provides the aquatic breeding environment required by these mosquitoes (Monath, 1994). Based on these demographic changes and subsequent increase in *Aedes* mosquito populations, Appawu *et al.* (2006) predicted an increase in DENV transmission in Africa. It has been suggested that susceptibility of different strains of *Aedes* species and populations to DENV varies geographically affecting DENV distribution in Africa (Diallo *et al.,* 2008) possibly driven by inherent and climatic factors (Wilson, 2000; Rogers & Randolph, 2006).

Vector competence (VC) is the intrinsic permissiveness of an arthropod vector for infection, replication and transmission of a virus (Alvaro *et al.,* 2005), mediated by the presence of several genetically determined barriers to viral transmission, including a midgut infection barrier (MIB) that prevents invasion and replication of the viruses and a midgut escape barrier (MEB) that prevents dissemination to other tissues (Black *et al.,* 2002). These barriers are major determinants of vector competence to DENV during experimental infections (Bennett *et al.,* 2002). The barriers also vary in prevalence in natural populations, leading to large intraspecific variation of *Ae. aegypti* vector competence and may determine the epidemiology of dengue viruses (Black *et al.,* 2002).

Susceptibility of different strains of *Aedes* spp. mosquitoes to DENV infection varies geographically and has implications for DENV distribution, transmission and epidemiology in Africa (Diallo *et al.,* 2008). Climatic factors are known to influence the incidence, seasonal variation, and global distribution of vector-borne diseases

(Rogers & Randolph, 2006). How these factors impact on the intensity of transmission of most vector-borne pathogens is not understood (Wilson, 2000). The ability to define the impact of climatic factors on the risk of vector-borne disease is limited by poor understanding of the link between environmental variables, such as temperature, and the vectorial capacity of insect vector populations (Paaijmans *et al.*,2009). Insects are small-bodied ectotherms, which makes their life cycle, survival and behavior dependent on the surrounding temperature (Tun-Lin *et al*.,2000).

Aedes aegypti is widely distributed across Kenya (Lutomiah *et al.,* 2013) and the risk of dengue virus transmission is therefore likely to be equally widespread. Dengue outbreaks have been reported multiple times at the Kenyan coastal towns of Malindi, Kilifi (Johnson *et al.,* 1982) and most recently Mombasa (KEMRI, 2012-2014), but despite the presence of *Ae. aegypti* in Nairobi, DENV transmission has not been documented. Whether these outbreaks are related to the urbanization and the area being a tourist destination is unclear. Like the coastal towns, Nairobi the capital city of Kenya is a major international hub hosting the largest airport in the East African region, facilitating the influx of a large number of international travelers. To explain the differences in outbreak occurrence between Nairobi and the Coast, this study hypothesized that the vectorial capacity of the *Ae. aegypti* populations between the two areas are different. While inherent differences between *Ae. aegypti* populations may exist, the contribution of differential ambient temperatures between these sites in driving the observed DENV transmission patterns has never been assessed. Therefore, this study sought to evaluate the vector competence of *Ae. aegypti* populations from Nairobi and Kilifi in laboratory experiments while incorporating the effect of prevailing ambient temperatures at these sites.

The occurence of dengue fever during the inter-epidemic periods has raised many questions on how the virus is maintained in nature (Johnson *et al.*,1982). Therefore, there is need to carry out studies to determine whether *Ae. aegypti* populations in Kilifi and Nairobi, Kenya can transmit DENV by transovarial transmission (TOT) or by vertical transmission (VT). The possibility of TOT suggests that early horizontal transmission of viruses to human increases (Mourya *et al.,* 2001), hence outbreaks.

These early horizontal transmission of viruses gives VT a role in the maintenance of the disease in nature, keeping virus levels even years before an outbreak occurs (Fouque *et al.,* 2004).

1.2 Statement of the problem

Dengue is a major public health problem worldwide, especially in the tropical and subtropical areas with around 3.5 billion people (55% of the world's population) living in areas at risk (WHO, 2009). In Africa, the epidemiology and public health effect of dengue is not clear. When *Aedes* species distribution is combined with rapid population growth, unplanned urbanization, and increased international travel, extensive transmission of DENV is likely in Africa (Gubler, 2004). Dengue has received little attention in the past in Kenya largely because the severe forms of dengue illness rarely occur in Africa (Sang, 2007). Dengue fever is often misdiagnosed and treated as malaria or other endemic fevers, such as typhoid and leptospirosis. This has resulted in underestimation of the magnitude of the dengue problem in Kenya. Dengue fever is now a re-emerging infection in Kenya where it has caused major outbreaks in parts of Northern Kenya and Coastal regions since 2011 and 2013 respectively. The widespread distribution of *Ae. aegypti* in Kenya poses a threat for dengue transmission. There is therefore the risk of introduction of DENV to Nairobi from the Northern and Coastal parts of Kenya through movement of people between the towns. On the other hand, the seasonality of dengue outbreaks could be attributed to other mechanisms such as vertical transmission by which DENV is maintained during inter-epidemic periods.

1.3 Justification

Dengue fever outbreak is one of the important public health problems in Kenya. Major outbreaks of dengue fever, mostly caused by dengue-2 virus, have occurred in Kenya (Coast and North Eastern) resulting in considerable morbidity and economic loss due to lost man hours (AFEN, 2013; KEMRI laboratory reports 2012-2014). The first outbreak of dengue in Kenya caused by dengue-2 virus was detected in 1982 (Johnson *et al.,* 1982) and since then the major subsequent outbreaks were detected from 2011 to date affecting parts of North Eastern and Coastal Kenya with Dengue

Serotypes 1-3 being detected (Unpublished data). Although dengue virus is one of the most important arbovirus affecting humans, there is no effective treatment other than supportive care with fluid management. Currently, most efforts to manage the disease are based on vector control (Kuno, 1997). Despite the presence of *Ae. Aegypti*, the principal vector across Kenya (Lutomiah *et al.*, 2013), DENV transmission has not been reported in Nairobi. It is therefore important to determine vector competence of Nairobi *Ae. aegypti* population in order to establish the level of risk of DENV transmission in the region. Both Nairobi and Kilifi Counties have different annual relative temperatures and it is not known if this has an effect on the selective transmission of DENV by the two *Ae. aegypti* populations. The movement of people between the two towns has the potential of introducing DENV into Nairobi and other parts of the country. Accurate data on vector competence of different populations of *Ae. aegypti* provides a better understanding of transmission dynamics and environmental factors associated with DENV transmission and hence facilitate designing effective preventive programs. No studies have been conducted to determine vertical transmission of DENV in Kenya. Therefore, it was necessary to study the vertical transmission in order to generate information that provides an understanding of the mechanism by which this virus is maintained in nature.

1.4 Hypothesis

1.4.1 Null Hypothesis

Variation in temperature has no effect on susceptibility and dissemination rates of DENV in *Ae. aegypti* mosquitoes.

1.5 Research Questions

1. What is the effect of rearing temperature on the susceptibility of Kilifi and Nairobi *Ae. aegypti* populations to dengue-2 virus?
2. What is the effect of rearing temperature on the dissemination of dengue-2 virus among Kilifi and Nairobi *Ae. aegypti* populations?
3. Does vertical transmission of dengue-2 virus occur among Kilifi and Nairobi *Ae. aegypti* populations?

1.6 Objectives

1.6.1 General objective

To determine the effect of rearing temperature on the vector competence of *Ae. aegypti* populations from Kilifi and Nairobi Counties for Dengue-2 virus

1.6.2 Specific Objectives

1. To determine the infection rates of dengue-2 virus in *Ae. aegypti* populations from Kilifi and Nairobi Counties under varying ambient temperature
2. To determine the dissemination rates of dengue-2 virus in *Ae. aegypti* populations from Kilifi and Nairobi Counties under varying ambient temperature
3. To determine the vertical transmission of dengue-2 virus among the Kilifi and Nairobi Counties *Ae. aegypti* populations

CHAPTER TWO

LITERATURE REVIEW

2.1 Dengue virus

Dengue viruses (DENV) belong to the genus *flavivirus* of the *Flaviviridae* family. They are mosquito borne and the principal vector (*Aedes aegypti*) is a day-biting domestic mosquito of public importance that breeds in natural or artificial water (Gubler, 2002). Dengue illnesses are caused by any of the four serologically related, but antigenically distinct viruses designated as DENV-1, DENV-2, DENV-3 and DENV-4 (Gubler, 1988). Other viruses in the *Flaviviridae* family of public health importance are Yellow fever, Japanese encephalitis and West Nile viruses. Infection with any one of these serotypes causes a mild, self limiting febrile illness (DF), however, a few cases develop severe life threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (Gubler & Clark, 1995).

2.1.1 Genome of dengue virus

The DENV particle is enveloped, with an icosahedral capsid that contains a single-stranded positive-sense RNA genome of approximately 11 kb that resembles a messenger RNA with a cap on the 5' end but no poly (A) tail at the 3' end (Chambers *et al.,* 1990). The envelope of DENV is composed of heterodimers of the (E) glycoprotein and the membrane (M) protein that are embedded in a host-derived lipid bilayer. The nucleocapsid is composed entirely of capsid (C) protein and encapsulates the RNA genome. The E glycoprotein is important for cell receptor attachment and subsequent infection of the target cell membrane, and bears the neutralization epitopes (Mukhopadhyay *et al.,* 2005). DENV, as well as all arboviruses, has evolved to replicate in the unique biochemical environments of both vertebrate and invertebrate hosts. As a result, the mature viruses are hybrid structures which derive their lipid bilayers from the host cell (Katherine *et al.*, 2011).

2.2 Infectivity of Dengue virus

Dengue is caused by four genetically related but antigenically different viruses, and although it is uncertain where DENV originated from, maintenance of all four serotypes in enzootic cycles in Africa suggests that a progenitor virus most likely originated in Africa (Gubler & Clark, 1995). Despite the apparent origin of DENV in Africa, the more recent reported outbreaks appear to be the result of virus introductions from Southeast Asia or the Western Pacific region and not the result of spillover from forest transmission cycles (Monath, 1994). The rate of evolutionary change and pattern of natural selection are similar among endemic and sylvatic DENV and there could be a possibility of future re-emergence of DENV from the sylvatic cycle (Vasilakis *et al.,* 2007). Recent experimental evidence suggests that emergence of endemic dengue-2 virus from sylvatic progenitors may not have required adaptation to replicate efficiently in humans, implying that sylvatic dengue-2 virus may re-emerge (Vasilakis *et al.,* 2007). Existence of a silent zoonotic transmission cycle affords a potential mechanism for emergence of dengue in human populations and for selection of virus variants with altered host range and vector relationships (Monath, 1994).

2.3 Host Susceptibility to Dengue virus

Viral factors have been shown to influence the manifestations of dengue virus in a host. According to a study by Vaughn (2000), higher blood viral load was detected in DHF patients in comparison to DF patients. Dengue-2 virus had a larger pleural effusion index than the other virus serotypes in Thailand (Vaughn *et al.,* 2000) and clearly related to the severe clinical forms in a study in Vietnam (Lan *et al.,* 2008). Imported to America, this genotype is thought to be the cause of the appearance of DHF in this region (Rico-Hesse *et al.,* 1997), providing additional proof for the higher virulence of the Asian dengue-2 virus genotype. Host susceptibility may also be determined by factors within the host after an exposure, plasma leakage happens often occurs at day 4-6 of fever when the viremia has already declined (Long *et al.,* 2009), revealing the role of host immunopathological mechanisms in disease severity. Both dengue-virus-infected monocytes with Antibody-Dependent Enhancement (ADE) phenomenon, and activated specific T lymphocytes are

responsible for the rapidly increased levels of cytokines in DHF (Halstead, 2008), which play a key role in inducing important clinical manifestations of DHF, that is, plasma leakage and shock. However, how the virus-host interaction causes the clinical outcome remains an important question. Epidemiological evidence indicates that host genetic factors are relevant and predispose the DHF/DSS development (Lan and Hirayama, 2011).

Host genetic factors influencing pathogenesis have also been suggested to account for some variability in susceptibility of DENV infection and disease expression among different races (Halstead *et al.,* 2001). During the 1981 and 1997 dengue epidemics in Cuba, blacks were hospitalized with DHF/dengue shock syndrome at lower rates than whites (Kouri *et al.,* 1989). This potential decreased susceptibility to severe disease among the black population and similar observations in Haiti have been used to support the hypothesis that specific genomic difference among different racial groups is a risk factor for DHF (de la C Sierra *et al.,* 2007).

2.4 Symptoms of Dengue virus infection

Dengue has four clinical manifestations, namely: undifferentiated illness, classic dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. Undifferentiated dengue, the most common syndrome, occurs when a DENV infection is asymptomatic or mildly symptomatic (Rigau-Perez *et al.,* 1998). Although classical dengue is not usually fatal it has very high morbidity, its alternate name is break-bone fever for the severe joint pain during infection. Disease is most common in infants, young children, and adults. Classical dengue manifests itself with a mild to high fever, red rash, debilitating headaches, muscle and joint pain lasting 2-7 days (George *et al.,* 1997). The more severe manifestations of the disease are dengue hemorrhagic fever and dengue shock syndrome. Young children and those previously infected with dengue are most at risk for these complications from dengue infection (Gubler & Clark, 1995). Dengue shock syndrome results when capillaries leak as result of infection. This leads to edema (swelling of tissue because of fluid escaping the circulatory system), abdominal pain (as a result of the edema) and hypotension (low blood pressure resulting from a drop in total blood volume from

fluid loss). Oxygen and nutrients stop reaching the body tissues because of inadequate circulation of the blood as a result of these symptoms and this can lead to shock and death. Dengue hemorrhagic fever occurs when normal blood coagulation is disrupted by infection. Fever, emesis, and bleeding are all symptoms common to this type of dengue. The results of internal hemorrhage can be seen in the form of tiny red spots (petechiae) or sometimes patches under the skin as well as bloody stool and bleeding from the gums and nose. Mortality from these complications can be up to 14% without proper care (Deubel *et al.*, 2001).

2.5 Diagnosis and treatment for dengue virus infection

The routine laboratory diagnosis of dengue virus infection is by the isolation of virus in tissue culture, serodiagnosis by detection of IgM/IgG antibodies and /or molecular detection by demonstration of viral RNA by RT-PCR (Yamada *et al.,* 1999). Dengue infection lacks an available and effective vaccine or drug treatment and effective warning parameters are needed to monitor the dengue situation, thus enabling an early outbreak response. Traditional dengue surveillance in most countries relies on case-based surveillance, laboratory-based surveillance, and surveillance of *Aedes* population densities (Supatra *et al.,* 2011). Monath (1989) and Gubler (2004) hypothesized that immunologic crossprotection from heterotypic antibodies to other flavivirus infections (DENV and Japanese encephalitis virus) could explain the absence of yellow fever virus in Asia. A similar argument could be made to explain the low rate of DENV infection caused by cross-protection from other endemic flaviviruses in Africa, but the extent to which it may exist is unknown.

2.6 Dengue virus vectors

Aedes aegypti mosquitoes, the principal DENV vector, originated from sub-Saharan Africa and spread to other countries in Africa and other tropical countries in the 17th and 18th centuries (Halstead, 2008). Several other *Aedes* species mosquitoes, including *Ae. albopictus*, *Ae. africanus*, and *Ae. luteocephalus*, are found in Africa and are potential DENV vectors. Urbanization is a major factor in facilitating the increase of *Aedes* species mosquito populations (Gubler & Clark, 1995).

Accumulation of non-biodegradable, human-made containers used to store water in and around living areas has provided the aquatic environment required by these mosquitoes (Monath, 1994). With these demographic changes and subsequent increases in *Aedes* mosquito populations, increased DENV transmission is likely to occur in Africa (Appawu *et al.,* 2006). Susceptibility of different strains of *Aedes* species mosquitoes to DENV has been shown to vary geographically, and this variability may have implications for DENV transmission and the epidemiology of the disease in Africa (Diallo *et al.,* 2008).

2.6.1 Global distribution and abundance of *Ae. aegypti* Mosquitoes

Originating sub-Saharan Africa (Halstead, 2008), *Ae. aegypti* probably invaded other continents through trading and transport ships that resupplied in African ports during the fifteenth to the seventeenth centuries. These ships carried freshwater reservoirs on board and could maintain breeding colonies of *Ae. aegypti* (Christophers, 1960), and it is probable that *Ae. aegypti* was introduced into the rest of the world on a number of occasions through this means (Tabachnick, 1991). Given the occurrence of concurrent outbreaks of dengue in Asia, Africa and North America in the late 1700s, it is probable that dengue vectors have had a wide a distribution throughout the world's tropics for the last two centuries (Gubler & Clark, 1995). However, the introduction of *Ae. aegypti* into Asia appears relatively recent, as endemic dengue fever in urban areas was not recorded in this region until the late nineteenth century (Smith, 1956), and low genetic diversity across tropical Asian populations suggests recent dispersal (Tabachnick, 1991).

Climatic factors alone do not determine the geographical distribution of *Ae. aegypti,* because the association of *Ae. aegypti* with domestic environments affords this species microenvironments which are highly moderated by human behavior and this in turn gives the mosquito the ability to avoid the effects of transient weather to some extent. The use of manually filled water receptacles obviates the mosquitoes' reliance on rainfall to flood suitable oviposition sites, and the tendency for adults to rest indoors provides shelter and protection from external environmental conditions (Jansen &Beebe, 2010). While *Ae. aegypti* larvae require water for survival, they readily utilise human-filled containers and thus are not totally dependent on rainfall

to flood their container habitats. The association between rainfall and *Ae. aegypti* abundance will almost certainly vary between localities due to both the range of container types that are available as larval habitats and differences in the water storage practices of the local population.

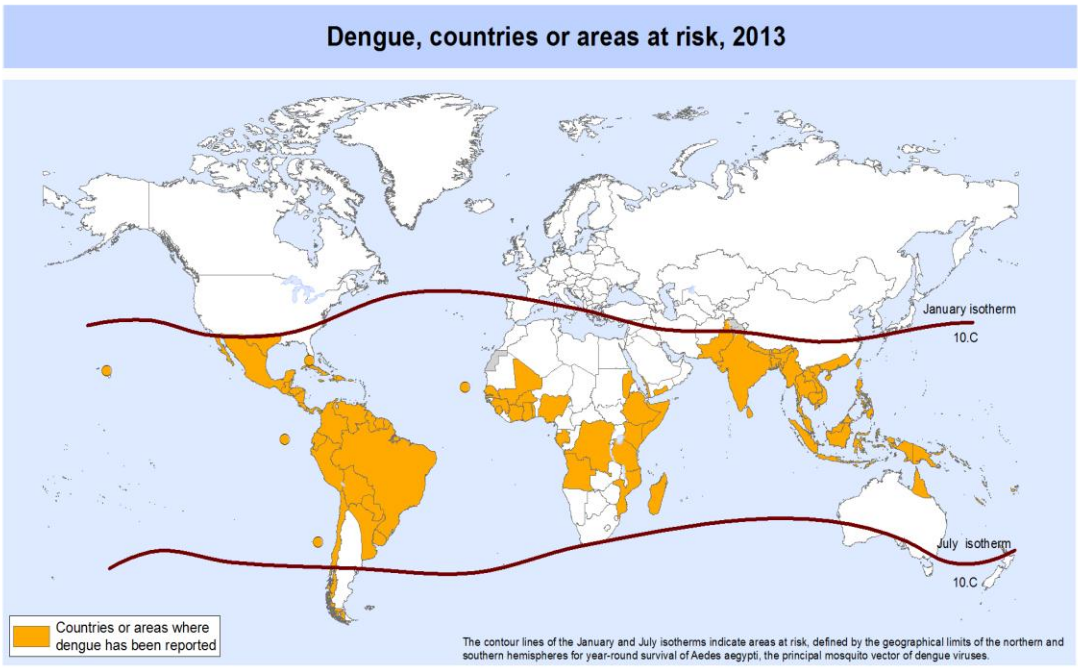
In most cases, it appears that the highly domestic nature of *Ae. aegypti* means that humans, rather than climate, drive the distribution of this species (Powell and Tabachnick, 2013). While local weather may explain the abundance of *Ae. aegypti* in a particular location at a given time, other factors may be more predictive for the presence of this species. Particular features that have been observed as associated with *Ae. aegypti's* presence include urbanization, socioeconomic factors, building design and construction features, the quality of water supply and management, and the quality of other public health infrastructure services (Monath, 1994).

The response to increased population size and density demands may dramatically alter the suitability of a locality for urban mosquito breeding. An absence or irregularity of water supply will lead to an increase in domestic water storage practices which, in turn, will alter the landscape of potential *Ae. aegypti* habitat perhaps providing a far more regular or numerous supply of larval sites (Jansen &Beebe, 2010). The potential increased risk of dengue re-emerging in Australia is attributable to human adaptation to the current and projected climate change scenarios which suggest the drying of southern Australia, rather than directly to changes in the climate (Beebe *et al.,* 2009). Indeed, it is the widespread installation of government subsidized rainwater tanks intended to drought-proof urban areas that may facilitate the re-appearance of *Ae. aegypti* in these regions. The degree of public health infrastructure in a community can also influence the abundance of *Ae. aegypti*. Poor sanitation standards which result in the accumulation of debris and other material that collect rainwater can facilitate major expansion of this mosquito's populations (Monath, 1994).

2.7 Global burden of dengue fever

Dengue is one of the notable viral infections, the global epidemiology of which has changed dramatically in the past 50 years especially during the last 20 years in the tropical regions of the world (Gubler, 1997). Dengue is a major public health

problem worldwide, especially in the tropical and subtropical countries with around 3.5 billion people living in areas at risk (WHO, 2009) as shown in table 2.1. Dengue has emerged in recent decades as a worldwide public health problem, particularly in the Asia-Pacific and Americas-Caribbean regions (Gubler & Clark, 1995). In Africa, the epidemiology and public health effect of dengue is not clear. When *Aedes* species distribution is combined with rapid population growth, unplanned urbanization, and increased international travel, extensive transmission of DENV is likely in Africa (Gubler, 2004). Over the past five decades, cases of epidemic or sporadic dengue have been reported in many countries in sub-Saharan Africa (WHO, 2009). However, when compared with the Asia-Pacific and Americas-Caribbean regions, the epidemiology and effect of dengue in Africa has not been defined

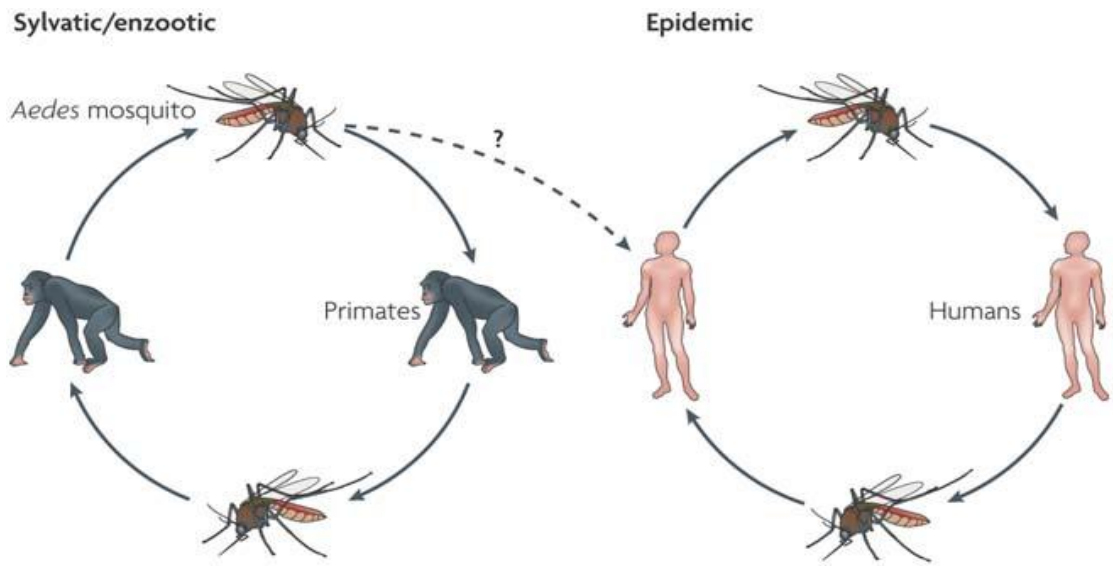


(Dengue/DHF update, 2009).

Figure 2.1: Dengue virus worldwide distribution map (WHO, 2014)

Dengue occurs in sylvatic cycles in Africa. Sylvatic vectors in Africa include *Aedes (Stegomyia) africanus*, *Ae. (Stegomyia) luteocephalus*, *Ae. (Stegomyia) opok*, *Ae. (Diceromyia) taylori*, and *Ae. (Diceromyia) furcifer* (Diallo *et al.*, 2003). These

sylvatic cycles, are believed to represent the ancestral DENV cycles from which epidemic/endemic strains of DENV-1- 4 evolved (Wang *et al.*, 2000). Although humans occasionally become infected with sylvatic DENV in Africa and perhaps in Asia, they are tangential to the maintenance cycle, which involves sylvatic *Aedes* species mosquito vectors and non-human primates as reservoir hosts (Figure 2.2).



Source: Nature reviews/ Microbiology

Figure 2.2: Dengue virus transmission cycles

2.7.1 Dengue in Eastern Africa

Although dengue epidemics are infrequent in Eastern Africa when compared with South-east Asia, the Americas and the Caribbean, all four serotypes of dengue have caused outbreaks in Eastern African region (Sang, 2007). In 1984-1985, an outbreak of dengue was reported in Mozambique where two deaths occurred (Gubler *et al.*, 1986). Another outbreak of dengue caused by dengue-2 virus was reported in the city of Djibouti, on the Horn of Africa, in 1991-1992, with cases of severe dengue being reported mainly among tourists and expatriates (Rodier *et al.*, 1996). In 1992-1993, an outbreak of dengue was reported among United States troops engaged in the mission Operation Restore Hope in Somalia, which lies to the north of Kenya. It was found that 2% of cases were caused by DENV-3 and 41% by DENV-2 (Sharp *et al.*, 1995). During the outbreak, only cases of classical dengue were seen, and there were

no cases of severe dengue. The available evidence so far indicates that DENV-1, -2 and -3 appear to be a common cause of acute fever in Eastern Africa, and that the frequency of epidemics continues to increase, with emergence of other serotypes since the Seychelles outbreak in 1977 (Metselaar *et al.*, 1980).

Although outbreaks and sporadic cases of dengue have continued to occur in eastern Africa, little effort has been made to identify vectors and transmission cycles (sylvatic, periurban or urban). It has been assumed that the outbreaks are most likely transmitted by *Aedes aegypti*, which is widely distributed in the region. Most of the reports on dengue in Eastern Africa are from outbreak investigations by visiting scientists and, in most instances, no entomological studies are performed and little serological surveys are done to determine the extent of the outbreaks.

2.7.2 Dengue in Kenya

In 1982, an outbreak of dengue fever caused by dengue-2 virus (DENV-2) was reported in the Kenyan Coastal towns of Malindi and Kilifi (Johnson *et al.*, 1982); clinical presentation was consistent with classical dengue fever, with no severe dengue reported. The 1982 outbreak in Kenya is believed to have spread from the Seychelles outbreak that occurred between 1977 and 1979 (Metselaar *et al.*, 1980). Since then there have been sporadic cases of dengue reported in Kenya and a serology survey carried out in 2005 revealed the occurrence of dengue transmission in coastal and inland parts of Kenya (Mease *et al.*, 2011). In 2012, dengue outbreak was reported in Northern Kenya and subsequently in the coastal town of Mombasa (AFEN, 2013; KEMRI, 2012-2014) and is now viewed as a re-emerging public health problem in Kenya. Together with WHO and Ministry of Health, surveillance studies are ongoing at the KEMRI to understand dengue virus vectors (Ligami and Buguzi, 2014).

2.8 Prevention of dengue virus

Promising candidate dengue vaccines are raising hopes of effectively preventing human disease (Pang, 2003). Humans are the only reservoir host for the endemic cycle, therefore an effective vaccine could ultimately eradicate endemic strains. This scenario underscores the need for greater understanding of the historical emergence

of human dengue from sylvatic origins to predict the facility with which the sylvatic strains could re-emerge to initiate urban transmission. One question to be answered is how many mutations are responsible for the efficient infection phenotype for *Ae. aegypti* and *Ae. albopictus* exhibited by the endemic dengue-2 virus strains. Identifying genetic determinants of DENV adaptation to these peridomestic vectors will ultimately provide an indication of the ability of these arboviruses to re-emerge.

The viral molecular determinants that confer DENV with the ability to infect and be transmitted by their mosquito vectors are not known. Phylogenetic studies suggest that the DENV E protein may be important in the adaptation to urban vectors. In particular, domain III of the E protein contains several hypothetical amino acid replacements associated with emergence of urban strains (Wang *et al.,* 2000). This clustering of changes in domain III is observed repeatedly during the emergence of DENV-1, DENV-2, and DENV-4, when phylogenetic methods are used (Cahour *et al.,* 1995). Another genomic region potentially important in mediating vector transmission may be the 5′ non-coding region. Deletions in this region of DENV- 4 constrain its ability to infect *Ae. aegypti* and *Ae. albopictus* mosquitoes (Moncayo *et al.*, 2004).

The increasing co-circulation of the four dengue virus types means that a tetravalent vaccine is needed that protects against the four serotypes. Four types of dengue vaccines are currently in development: live attenuated virus vaccines, chimeric live attenuated virus vaccines, inactivated or sub-unit vaccines, and nucleic acid-based vaccines. Several DNA vaccines designed to deliver structural dengue viral genes into cells have been generated, and a monovalent DENV-1 DNA vaccine is currently undergoing phase 1 testing. In addition, candidate vaccines that have successfully protected monkeys from viraemic challenge include recombinant 80% envelope protein from the four DEN serotypes in conjunction with DENV-2 NS1 administered with several new-generation adjuvants. There is also work in progress on subunit vaccines based on domain III of the E protein, which is considered to be the principal neutralizing epitope region of the virus, employing different strategies to increase immunogenicity. Since dengue is caused by four serologically related viruses, a major problem in developing a dengue vaccine is to develop four immunogens that

will induce a protective immune response against all four viruses simultaneously. Therefore, the vaccine must be tetravalent. Interference between the four vaccine viruses must be avoided or overcome, and neutralizing titres to all four viruses must be achieved regardless of the previous immune status of the vaccinated individuals (WHO, 2009). Given the complexity of dengue and dengue vaccines, it is important to continue with scientific research that is directed at improving the understanding of the immune response in both natural dengue infections and vaccines (e.g. defining neutralizing and potentially enhancing epitopes, improving animal models) alongside vaccine development and evaluation.

2.9 Viral vector competence studies

Vector competence (VC) is the intrinsic permissiveness of an arthropod vector for infection, replication and transmission of a virus (Alvaro *et al.,* 2005). Overall VC is mediated by the presence of several genetically determined barriers to viral transmission, including a midgut infection barrier (MIB) that prevents invasion and replication of the viruses and a midgut escape barrier (MEB) that prevents dissemination to other tissues (Black *et al.,* 2002). These barriers are major determinants of vector competence to DENV during experimental infections (Bennett *et al.,* 2002). The barriers also vary in prevalence in natural populations, leading to large intraspecific variation of *Ae. aegypti* vector competence and may determine the epidemiology of dengue viruses (Black *et al.,* 2002).

The first step in infection of a mosquito as a result of ingestion of a viremic bloodmeal is infection of the midgut epithelial cells, and passage of the midgut infection barrier (MIB) requires the virus to attach, penetrate and replicate in the midgut epithelial cells. Following the successful establishment of infection in the midgut epithelial cells, the infectious virions generated must overcome the midgut escape barrier (MEB) which involves passing through the basal lamina and the haemocele in order to establish infection in secondary target organs (Black *et al.,* 2002).

Studies on the flaviviral vector competence of *Aedes* mosquitoes have suggested that the midgut infection barrier is the major determinant of transmission (Bosio *et al*., 1998). Many studies have focused on determining genetic differences in mosquitoes

from a wide geographic distribution. More recently, significant genetic variation in *Ae. aegypti* on a smaller scale has been demonstrated in Puerto Rico and in Mexico (Bennett *et al.*, 2002).

2.10 Vertical transmission of Dengue virus

Transovarial transmission (TOT) of dengue virus infection has been reported in *Aedes* mosquito vectors and has become a topic of research interest in many endemic countries around the world (Thongrungkiat *et al.*, 2011). Vertical transmission is a mechanism in which the virus is transmitted from an infected female mosquito to her eggs as they pass through the genital tract. This mechanism is known to support the persistence of some arboviruses in nature (Rosen, 1988) as a possible mechanism of surviving long period of adverse climatic conditions in *Aedes* transmitted arboviruses. *Ae. aegypti* mosquitoes are able to transmit the Dengue virus vertically, or transovarially, to their offspring either by oral feeding or intrathoracic inoculation under laboratory conditions (Joshi *et al.*, 2002; Wasinpiyamongkol *et al.*, 2003). In addition, transovarial transmission has been reported in nature by the detection of the dengue virus in the field-collected larvae and field caught adult male mosquitoes (Guedes *et al.*, 2010). Therefore, transovarial transmission (TOT) has been suggested as a potential influence on the epidemiology of dengue infection. It has been proposed as an important mechanism for the maintenance of the dengue virus in vector populations during inter-epidemic periods (Rhodain and Rosen, 1997). Integrated vector management uses well established methods for suppressing the vector populations to prevent and control dengue outbreaks. However, improved knowledge of dengue transmission and effective pro-active surveillance systems are needed to improve dengue prevention and control strategies.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study sites included the Coastal and Central parts of Kenya (Figure 3.1). At the coast, eggs of *Ae. aegypti* mosquitoes were collected from Bengo village, Mbarakani village and Changombe Sub-location in Rabai sub County, in Kilifi County based on prior history of dengue circulation in the area (Johnson *et al.,* 1982). Kilifi County (latitude of 3.63°S and a longitude of 39.85°E) has a mean daily temperature of 30°C, rainfall of approximately 88.25 mm and 82% relative humidity. Collections in Nairobi were from Githogoro village in Westlands sub County, Karioko village in Starehe sub County and Karura forest, all in Nairobi County (latitude of 1.28°S and a longitude of 36.81°E) with a mean daily temperature of 26°C, rainfall of approximately 85.35 mm and 70% relative humidity.

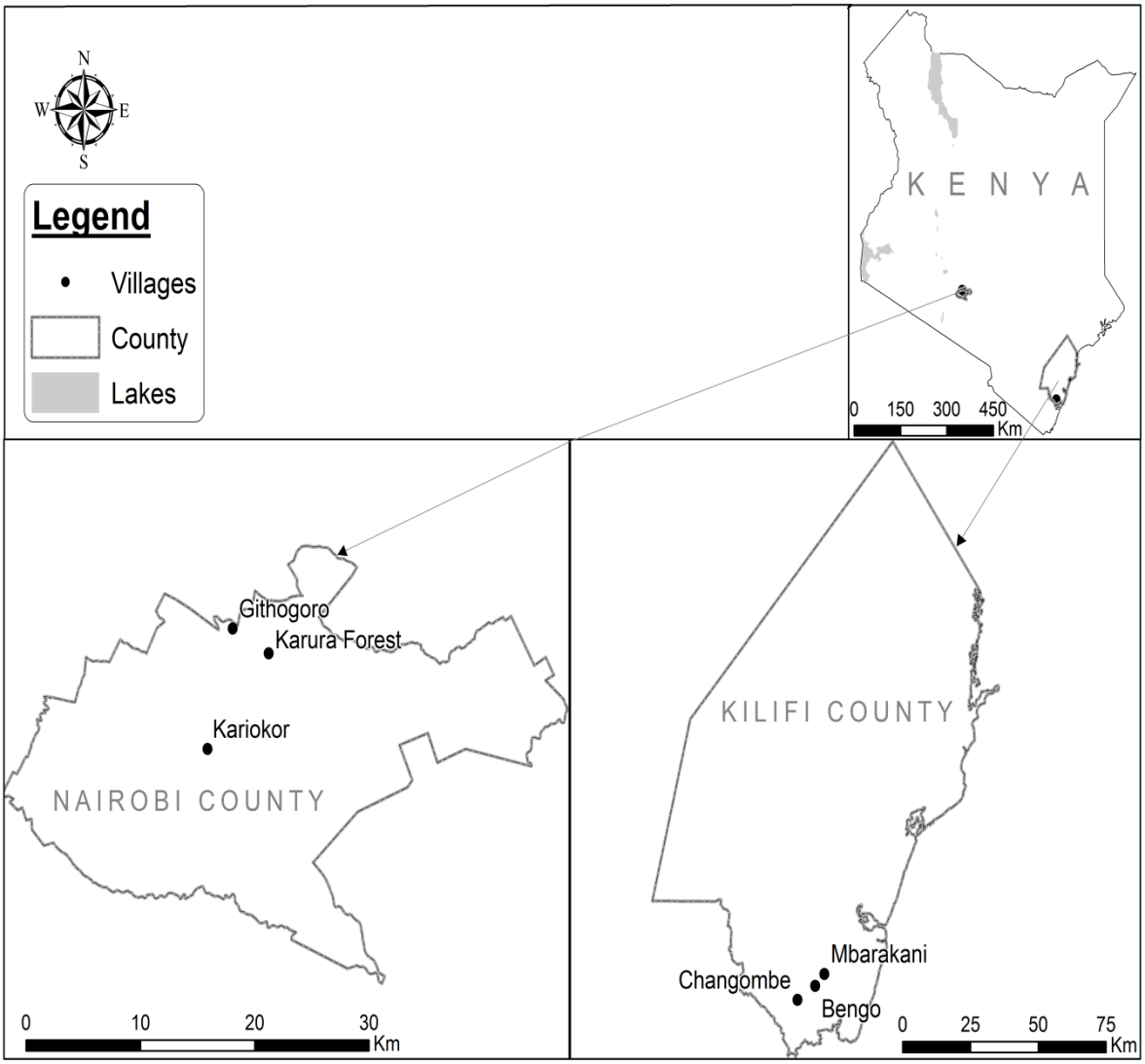


Figure 3.1: Map showing the study area and the mosquito eggs collection sites

3.2 Study design

The study adopted a laboratory-based experimental design.

3.3 Mosquito egg collection

Aedes aegypti eggs were collected using oviposition cups (ovicups), lined with oviposition papers and half-filled with water. These were set at various points, at least 100 meters from each other, in the study area for 4 days with the Global Positioning System (GPS) coordinates of each ovicup point taken for geo-referencing. On day 4, all the ovicups were collected, the eggs on the Oviposition papers were folded and place in zip lock bags, and then transported to the biosafety

level-2 (BSL-2) insectary in the viral hemorrhagic fever laboratory at the Kenya Medical Research Institute (KEMRI) in Nairobi, where they were dried on damp cotton wool to quiescent state (Steinly *et al.*, 1991), and stored in an air tight container at room temperature in the insectary.

3.4 Mosquito rearing

A total of 456 eggs were collected from the Kilifi County sites and 324 eggs from the Nairobi County sites. As needed, several batches of eggs (F_0) from Kilifi and Nairobi areas were dispensed in water on larval trays for hatching in the KEMRI level 2 insectary, maintained at a temperature of 28°C and 80% relative humidity (RH), with a 12:12-hour (Light:Dark) photoperiod. The larvae were fed on yeast mixed with tetramin fish food until they pupated. The pupae were collected every morning and put in a cup containing water. The cup with pupae was placed in a 1-gallon plastic cage with a netting material on top and allowed to develop into F_0 adult mosquitoes. The emerging adults were knocked down by placing them in a -20°C freezer for one minute, then morphologically identified under a dissecting microcope using taxonomic keys of Edwards (1941), to ensure that only *Ae. aegypti* mosquitoes were used in the subsequent experiments. Eggs collected from Kilifi County hatched into 370 mosquitoes of which 237 were identified as *Ae. aegypti* and for Nairobi County sites, eggs hatched into 288 mosquitoes of which all were identified as *Ae. aegypti*. Positively identified *Ae. aegypti* mosquitoes were returned to their experimental cages, blood fed on clean laboratory-bred mice and provided with oviposition papers to lay F_1 eggs. The F_1 eggs were hatched and reared as decribed above. Only adult female mosquitoes were used in the subsequent experiments (Gerberg, 1970).

3.5 Amplification of dengue-2 virus

Dengue virus serotype 2 (DEN-2) which was isolated from a patient's sample (Sample number: 008/01/2012) from the 2012 dengue outbreak in Mandera, Kenya, was used in the study. The virus was passaged in T-75cm^2 culture flask containing C6/36 cell lines (*Ae. albopictus* mosquito cell lines), grown in Dulbecco's modified eagles medium (DMEM), (GIBCO® Invitrogen corporation, Carlsbad, California), liquid (4.5 g/L D-glucose) without L-glutamine and sodium pyruvate, supplemented

with 10% heat-inactivated fetal bovine serum (FBS), (Sigma-Aldrich, St. Louis, MO), 2% L-Glutamine (Sigma-Aldrich, St. Louis, MO), and 2% antibiotic/ antimycotic solution with 10,000 units penicillin, 10mg streptomycin and 25µg amphotericin B per ml (Sigma-Aldrich, St. Louis, MO) and incubated at 28°C in 5% CO_2 over night. Confluent monolayer of C6/36 cells were inoculated with 600µl of the dengue virus supernatant isolate and incubated for 1 hour with frequent agitation/rocking to allow for virus adsorption. The infected cells were maintained in DMEM supplemented with 2% FBS, 2% L-Glutamine and 2% antibiotic/ antimycotic, incubated at 28°C in 5% CO_2 and observed daily for cytopathic effect (CPE) for a period of 7 days. Once the CPE was observed to affect 80% of the monolayer, flask was frozen overnight at -80°C, thawed on wet ice, then clarified by centrifugation at 3000 revolutions per minute for 10 minutes and the supernatant harvested by aliquoting into 1.5ml cryovials. All the aliquots were stored at -80°C (Gubler *et al.,* 1984).

3.6 Quantification of dengue-2 virus

Quantification of dengue-2 virus was performed by plaque assay. Ten fold serial dilutions of the amplified DENV was done and inoculated in 6 well plates containing confluent Vero monolayers as described by Gargan *et al.* (1983). This was 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 (FBS), (Sigma-Aldrich, St. Louis, MO), 2% L-Glutamine (Sigma-Aldrich, St. Louis, MO), and 2% antibiotic/ antimycotic solution with 10,000 units penicillin, 10mg streptomycin and 25µg amphotericin B per ml (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C in 5% CO_2 over night. Each well was inoculated with 100µl 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 9 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 (Gargan *et al.,* 1983):

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

where d is the dilution factor and V is the volume of diluted virus added to the well. The titer for dengue-2 virus used in this study was calculated as:

Number of plaques = 12 plaques

Dilution factor (d) = 10^{-3}

Volume of diluted virus inoculated (V) = 100µl (10^{-1})

$$\frac{\mathbf{12}}{\mathbf{10-3\ X\ 10-1}} = 1.2 \text{ X } 10^5 \text{ pfu/ml}$$

$$= \underline{10^{5.08} \text{ pfu/ml}}$$

3.7 Vector competence studies

3.7.1 Mosquito infection with Dengue virus

An infective blood meal was prepared by mixing DEN-2 virus stock with a virus titer of $10^{5.08}$ plaque-forming units (PFU)/ml and defibrinated sheep blood, in a ratio of 1:1, to end up with $10^{3.03}$ PFU/ml virus concentration. The virus/blood mixture was put in membrane feeders covered with a freshly prepared mouse skin (Higgs *et al.*, 1996), and maintained using the Hemotek system which employs an electric heating element to maintain the temperature of the blood meal at 35°C±1°C (Cosgrove *et al.*, 1994). Four-day-old adult female mosquitoes were allowed to feed on the infectious blood meal through the mouse skin for 1hour. After feeding, fully engorged mosquitoes were selected and put in secured cages, where they were maintained on 8% glucose for 7 to 21 days at set temperatures and 80% relative humidity. The first set of experiment involved exposing both Kilifi and Nairobi mosquitoes to temperatures set at an average of 26°C (similar to Nairobi temperature conditions). The second set of experiment involved exposing another group of mosquitoes from both sites to temperatures set at an average of 30°C (similar to Kilifi temperature conditions). Mortality was monitored in the cages by removing and counting dead mosquitoes daily (Cosgrove *et al.,* 1994).

3.7.2 Test for susceptibility of *Ae. aegypti* to Dengue virus

After every 7 days up to day 21 of incubation, 30% of live exposed mosquitoes were randomly picked and each dissected to separate the abdomens and legs (Table 3.1). Each mosquito abdomen was placed separately in 1.5ml eppendorf tubes containing 500µl of homogenization media (HM), consisting of MEM, supplemented with 15% FBS, 2% L-Glutamine, and 2% antibiotic/ antimycotic. The individual abdomens were homogenised using plastic grinders and the supernatant diluted in 10 fold serial dilutions. The dilutions were inoculated in confluent Vero cell lines in 12-well plates, grown in MEM, supplemented with 10% FBS, 2% L-Glutamine and 2% antibiotic/ antimycotic. The infected cell monolayers were then overlaid with methylcellulose supplemented with 2% FBS, 2% L-Glutamine and 2% antibiotic/ antimycotic and incubated at 37°C in 5% CO_2. On day 9, 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 plaques formed were counted and calculated using the formula described in 3.6 by Gargan (1983) for each mosquito abdomens to quantify the virus in the abdomens in order to determine the rate of infection (Turell *et al.*, 1984).

Table 3.1: The number of mosquitoes sampled after every 7 days

Temp. condition	Total no. sampled (30%)	Days post Infection		
		7	14	21
Nairobi Mosquitoes				
26° C	249	88	82	79
30° C	268	88	89	91
Kilifi Mosquitoes				
26° C	300	100	100	100
30° C	300	100	100	100

3.7.3 Test for Dissemination rates of Dengue virus

For each positive abdomen, corresponding legs were homogenised and their infection status determined as described in 3.7.2. Plaques were counted and calculated using the formula described in 3.6 by Gargan (1983) for each mosquito legs, to determine the dissemination rates (Turell *et al.*, 1984).

3.8 Determination of vertical transmission of Dengue virus

Adult female mosquitoes from the two different regions were exposed to an infective blood meal through membrane feeding. Each infected mosquito was put in a 1-pint cage and provided with oviposition paper on day 4 for the mosquito to lay eggs on (1^{st} gonotrophic cycle). The eggs were collected (676 eggs for Nairobi and 457 eggs for Kilifi mosquitoes) and dried gradually on damp cotton wool. On day 8 the same mosquito was fed on a clean mouse and the mouse euthanized immediately. The 2^{nd} gonotrophic cycle eggs were collected (342 eggs for Nairobi and 739 eggs for Kilifi mosquitoes) on day 12 and the mosquitoes allowed to have a 3^{rd} blood meal from a clean mouse on day 16 and eggs collected after 4 days (282 eggs for Nairobi and 591 eggs for Kilifi mosquitoes). The eggs collected at every cycle were used to determine the stage and the cycle at which VT of dengue virus takes place. All the eggs collected from the different cycles were hatched and reared under normal insectary conditions at 28°C and 80% RH. Adult mosquitoes from one parent were pooled into a maximum of 10 mosquitoes per pool, homogenized and inoculated in Vero cell lines. Plaque assay was carried out to confirm transovarial transmission (Turell *et al.*, 2008).

3.9 Laboratory safety considerations

Full personal protective equipment (PPE) was used when performing all the experiments. The infected mosquitoes were reared in double cages and in a well secured level-2 insectary at KEMRI. The virus was manipulated in a biosafety cabinet level-2 (BSC-2). Infected sharps were disposed in a sharps container. The

infected blood meal was inactivated in 10% bleach, before incineration. Animal carcasses were put in biohazard bags and transported for incineration.

3.10 Data analysis

Analyses were performed using R version 2.15.1 (R Core Team, 2012). Data on *Ae. aegypti* mosquito infection and dissemination rates at the two temperature levels and/or sites were compared using Chi square (and Fisher's exact) test at 5% significance level and confidence intervals (CIs) for the proportions estimated (Kirkwood and Sterne, 2003; Newcombe, 2012). The occurrence and extent of dengue virus vertical transmission (per 1,000 *Ae. aegypti*) was computed using PooledInfRate (Version 3.0), A Microsoft Excel plug-in (Biggerstaff, 2006).

3.11 Ethical Considerations

Scientific and ethical approval was obtained from the Kenya National Ethical Review Committee at the Kenya Medical Research Institute (Appendix 2) Animal use approval was obtained from the KEMRI Animal Care and Use Committee (ACUC).

CHAPTER FOUR

RESULTS

4.1 Susceptibility to Dengue- 2 virus infection in mosquitoes from Nairobi and Kilifi Counties

Dengue-2 virus infection rates were measured in a total of 517 *Ae. aegypti* female mosquitoes in three replicates from Nairobi region (249 under low and 268 under high temperature). Of these, 87 mosquitoes (16.8%; 95%CI: 13.7-20.3%) had DENV-2 infection in the midgut. The proportion of Nairobi mosquitoes with midgut infection was significantly higher in high temperature (21.3%) than at low temperature (12.0%; P=0.0037). Figure 4.1 presents the percentage of infected mosquitoes at day 7, 14 and 21 post-infection for each temperature level with the highest infection recorded on day 14 post-infection at high temperature of 30º C.

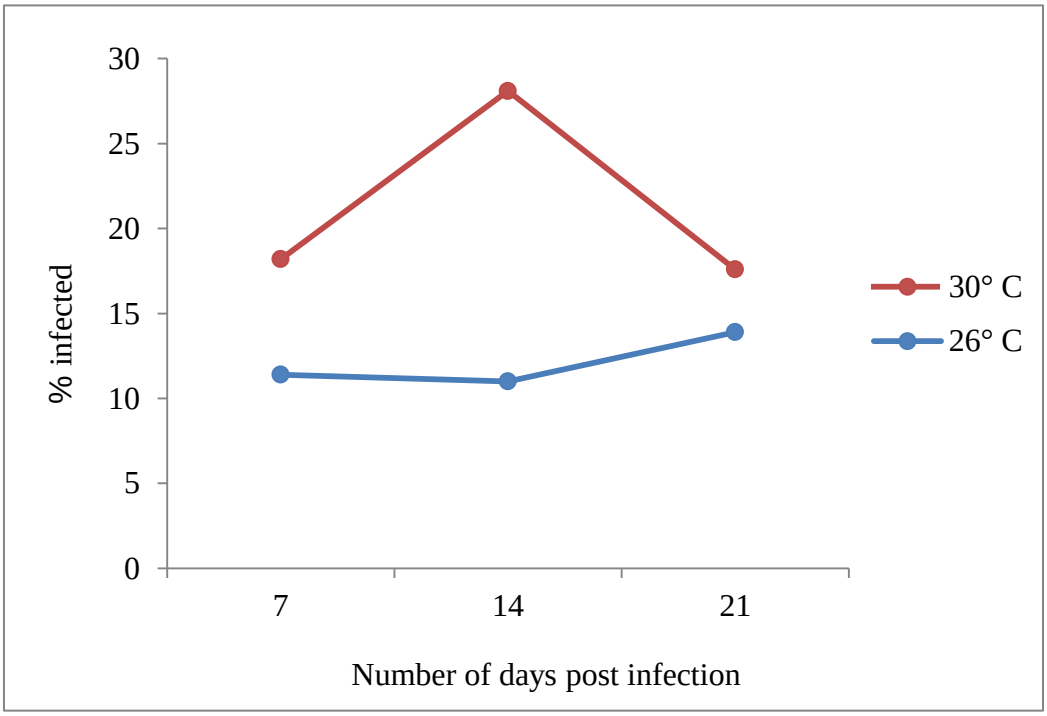


Figure 4.1: Proportion of Nairobi Ae. aegypti population infected at day 7, 14 and 21 post infection with DENV for each temperature level.

Kilifi mosquito population infection rates were measured in a total of 600 *Ae. aegypti* female mosquitoes in three replicates (300 in low and 300 in high temperature regimes). Of these, 54 mosquitoes (9%; 95%CI: 6.8-11.6%) developed midgut infection, with a significantly higher proportion recorded at high temperature (11.6%) relative to low temperature (6.8%; P=0.0162). The percent of infected mosquitoes at day 7, 14 and 21 post-infection for each temperature level is presented in Figure 4.2. There were low infection rates at 14 days post-infection under high temperature, with the highest infection at day 21 for both temperature levels Figure 4.2.

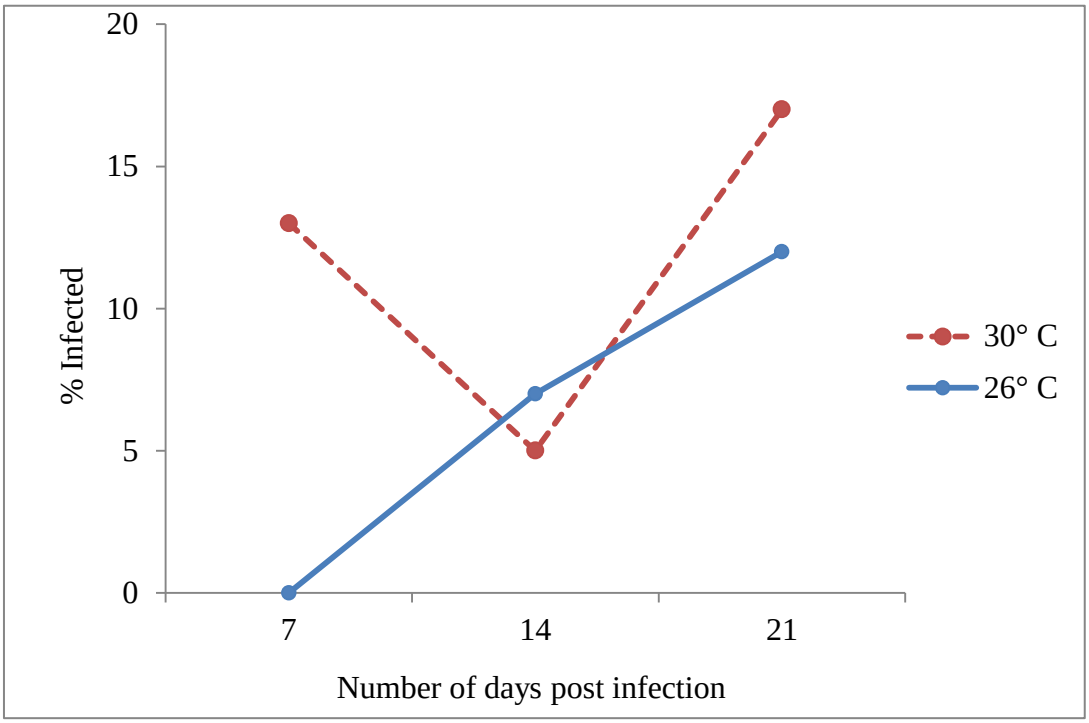


Figure 4.2: Proportion of Kilifi *Ae. aegypti* population infected at day 7, 14 and 21 post infection with DENV for each temperature level.

Overall data for both Nairobi and Kilifi mosquito populations showed that 141 of the 1,117 mosquitoes (12.62%; 95% CI: 10.73-14.71%) had midgut infection with a significantly higher proportion recorded in mosquitoes from Nairobi (16.8%) than those from Kilifi (9%; χ^2 =14.73, df=1, P=0.0001). Analysis from both experiments showed that mosquito infection was significantly influenced by temperature

(P<0.001) with higher infections recorded for mosquito populations from Nairobi relative to Kilifi at both temperature levels (Figure 4.3).

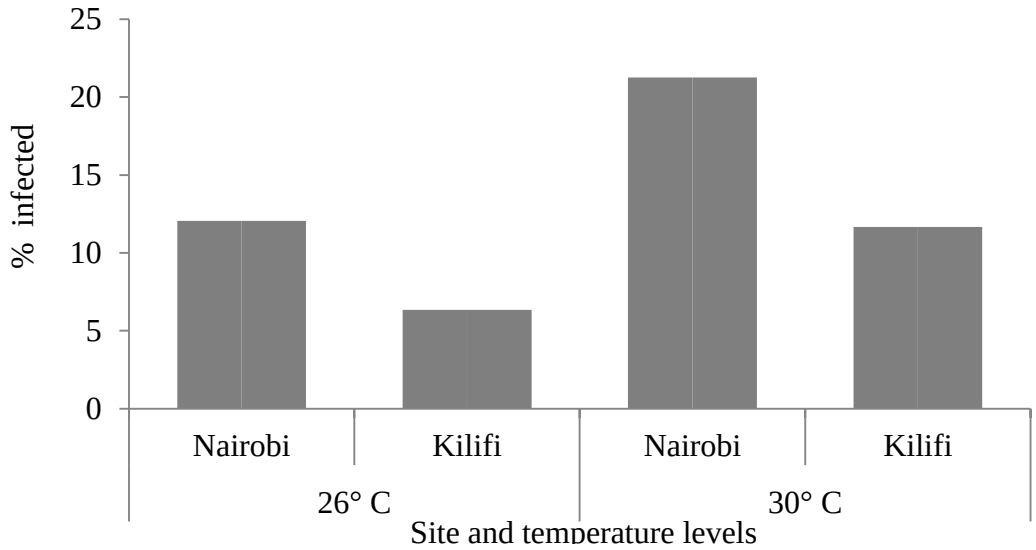


Figure 4.3: Mosquitoes infected with DENV per site at each temperature level.

4.2 Dissemination of Dengue-2 virus in mosquitoes from Nairobi and Kilifi Counties

The proportion of mosquitoes with disseminated infection was significantly higher for Kilifi mosquito population (40.7%; 95%CI: 27.6-55.0%) relative to that from Nairobi (10.3%; 95%CI: 4.8-18.7%; P<0.0001). This was evident at both the low and high temperature levels (Figure 4.4). Figure 4.4 shows that, while the highest dissemination was observed for Kilifi mosquito population at the high temperatures, the lowest was recorded for Nairobi mosquito population.

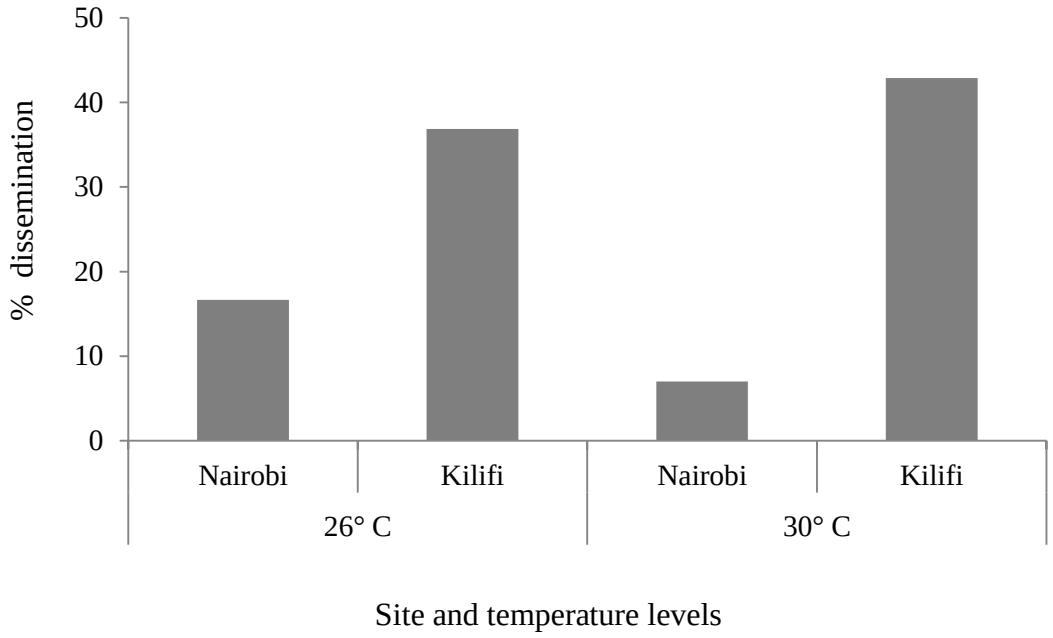


Figure 4.4: DENV infected mosquitoes with disseminated infection per site at each temperature level.

The results further showed that, for Nairobi mosquito population, a higher disseminated infection was recorded at low (16.7%, n=30) relative to high temperature (7.02%, n=57) although the difference was not significant (P= 0.2648). There was a negative association between temperature and the dissemination of DEN-2 virus by the Nairobi mosquito population. In contrast for Kilifi mosquito population, a lower dissemination rate was recorded at low (36.84%) compared to at high temperature (42.86%) although the difference was not significant (χ^2= 0.0195, df= 1, P= 0.889).

4.3 Vertical transmission of DENV among the Kilifi and Nairobi *Ae. aegypti* populations

The highest dengue virus pooled infection rate was observed for mosquitoes from Kilifi at a temperature of 26°C (160 per 1,000 *Ae. aegypti*), followed by Nairobi at a temperature of 30°C (135.71 per 1,000 *Ae. aegypti*), then Kilifi at 30°C (85.43 per 1,000 *Ae. aegypti*) and Nairobi at 26°C showing the lowest pooled infection rates of 60.98 per 1,000 *Ae. aegypti*. Further, it can be seen that other than Kilifi at a

temperature of 26°C (where it occurred at the 2nd Gonotrophic Cycle), the transmission of DENV-2 occurred during the 1st gonotrophic cycle (GC) only. The pooled infection rates at 1st, 2nd and 3rd gonotrophic cycles separately and for all the three GC combined are summarized in Table 4.1.

Table 4.1: Transovarial transmission (TOT), Pooled Infection Rates at 1st, 2nd and 3rd gonotrophic cycles (GC) separately and combined by temperature and site (viral titer 10 3.03 PFU/ml).

Site	Temp.	Pool details	1ST GC	2ND GC	3RD GC	All GCs combined
Nairobi	26°C	Pool size	2	2	2	
		# pools tested	27	22	33	82
		# infected pools	5	0	0	5
		Pooled IR	185.19	0.00	0.00	60.98
	30°C	Pool size	10	7	5	
		# pools tested	60	40	40	140
		# infected pools	19	0	0	19
		Pooled IR	316.67	0.00	0.00	135.71
Kilifi	26°C	Pool size	5	3	3	
		# pools tested	40	40	45	125
		# infected pools	17	3	0	20
		Pooled IR	425.00	75.00	0.00	160.00
	30°C	Pool size	4	8	7	
		# pools tested	61	75	63	199
		# infected pools	17	0	0	17
		Pooled IR	278.69	0.00	0.00	85.43

Pooled IR: Pooled Infection Rate= $\left(\left({}^{\#\text{ infected pools}}/_{\#\text{pools tested}}\right) \text{x}1{,}000\right)$

CHAPTER FIVE

DISCUSSION

5.1 Susceptibility in *Ae. aegypti* populations from Nairobi and Kilifi Counties to Dengue -2 virus infection under varying ambient temperature

This study constitutes the first initial effort to understanding the role of *Ae. aegypti* in driving dengue-2 virus transmission in Kenya, to assess the risk of further spread of the disease by analyzing the vector competence of *Ae. aegypti* populations from parts of Kenya and the influence of environmental factors like temperatures that prevail in outbreak hotspots and other areas of potential risk. The findings from this study show that *Ae. aegypti* from Nairobi were more susceptible to dengue-2 virus infection compared to the Kilifi mosquito population. This variation in *Ae. aegypti* populations to DENV susceptibility has been reported in various studies (Gubler *et al.,* 1979; Knox *et al.,* 2003). A study done by Moncayo (2004) on mosquito populations from various geographical locations showed that *Ae. aegypti* from Galveston, Texas, were more susceptible than those from Bolivia, but were less susceptible than mosquitoes from Thailand. Similar observations were made by Bennett *et al.* (2002) on *Ae. aegypti* collections from various locations in Mexico which differed significantly in their midgut susceptibility to infection.

The results of this study demonstrate significantly higher infection rates of dengue-2 virus at high temperatures for *Ae. aegypti* mosquitoes from both Nairobi and Kilifi, which is consistent with results by Watts (1987). This observation suggests a potentially significant role of temperature in the dynamics of DENV transmission. Studies have shown that temperature is one of the most important factors affecting biological processes of mosquitoes including their interaction with viruses (Watts *et al.,* 1987; Lambrechts *et al.,* 2011).

Kilifi County experiences high annual temperature (average of 30°C), rainfall (average of 88.25mm) and relative humidity (82%) as compared to Nairobi County which could explain why there have been several dengue fever outbreaks in the

region. Environmental conditions play a major role in infection of viruses. High environmental temperature increases virus multiplication to high titers and reduces the extrinsic incubation period for the virus to be established within the vector (Lambrechts *et al.,* 2011); get to the salivary glands after which it can be transmitted to another host (Watts *et al.,* 1987). Relative humidity on the other hand enhances mosquito survival allowing it to survive long enough for virus extrinsic incubation period to be complete allowing for virus transmission.

5.2 Dissemination of Dengue-2 virus in *Ae. aegypti* populations from Nairobi and Kilifi Counties under varying ambient temperature

The proportion of mosquitoes with disseminated dengue-2 virus infection was significantly higher for Kilifi mosquito population relative to that from Nairobi ($P<0.0001$). This was evident at both 26°C and 30°C temperature levels. The findings also show that, while the highest dissemination was observed for Kilifi mosquito population at the high temperatures, the lowest was recorded for Nairobi mosquito population, a finding which contrasts the pattern observed with midgut infection rates, in which the highest rates were observed in the Nairobi mosquitoes for dengue-2 virus. The results further showed that, for Nairobi mosquito population, a higher disseminated infection was recorded at low relative to high temperature although the difference was not significant ($P= 0.2648$). There was a negative association between temperature and the dissemination of dengue-2 virus by the Nairobi mosquito population. In contrast for Kilifi mosquito population, a lower dissemination rate was recorded at low temperature compared to at high temperature although the difference was not significant ($P= 0.889$).

Both Kilifi and Nairobi *Ae. aegypti* populations were incubated at conditions that mimic the environmental conditions of both sites, but still low disseminated infection was observed in the Nairobi population. Like several earlier studies (Watts *et al.,* 1987; Lambrechts *et al.,* 2011), these findings highlight the need for a better mechanistic understanding of the environmental determinants of vector pathogen interactions. Our findings are similar to these studies', in that the midgut and

disseminated infection rates differed significantly with *Ae. aegypti* populations from the two different sites, even after exposure to the same conditions in the laboratory. Disseminated infection for many mosquito-borne viruses is known to be affected by temperature, and transmission of an arthropod-borne pathogen is only possible where there is disseminated infection (Vazeille *et al.,* 2003).

There are a number of factors that contribute to the vectorial capacity of a mosquito for an arbovirus, including mosquito survival, density, proportion of infected mosquitoes that are feeding, length of extrinsic incubation period, vector susceptibility to the virus, and density of susceptible hosts (Moncayo *et al.*, 2004). This study investigated *Ae. aegypti* vector susceptibility to midgut infection and ability to disseminate dengue-2 virus, as a measure of the extent of adaptation of a virus to the vector and its potential to transmit it. However, the full competence of a vector is established by both its ability to become infected and to transmit the virus (Turell *et al.,* 1984). This parameter gives vector competence its epidemiologic importance. In this study, transmission potential was estimated from dissemination rates because previous studies have suggested that mosquitoes are capable of transmitting DENV as long as the virus is able to disseminate from the midgut into the hemocoel subsequently finding its way to the salivary glands for transmission (Gubler *et al.,* 1979; Vazeille *et al.,* 2003). An assumption was made based on these previous studies that mosquitoes that have a disseminated infection were capable of transmitting.

The overall findings from this study demonstrate that the Nairobi *Ae. aegypti* population is a relatively inefficient vector for DEN-2 virus compared to that from Kilifi with Nairobi population depicting high infection, but low dissemination rates in both low and high temperature settings. These findings suggest a weak midgut infection barrier (MIB) and a strong midgut escape barrier (MEB) for the Nairobi population and a moderate MIB, but weak MEB for the Kilifi population. The Kilifi *Ae. aegypti* population demonstrated inherent capacity to be more efficient in Dengue-2 virus transmission than the Nairobi population. Overall, the vector competence is considered as being inversely proportional to the level of MIB and MEB in the mosquito population: strong MIBs and MEBs reduce the potential for

infection and dissemination and eventually transmission by the vector (Bennett *et al.,* 2002). This could explain why there has been no evidence of active dengue transmission in Nairobi despite reported cases of dengue in health facilities usually from individuals who have travelled from Mombasa (coast) and/or Mandera (North-eastern Kenya) where outbreaks have been reported.

Aedes aegypti is reported to have originated in Africa, adapted to the peridomestic environment of African villages before being exported to America by slave trade and elsewhere by commercial transportation (Gubler, 1997). Mombasa at the Kenyan Coast is the second largest city of Kenya and a major port. It could therefore serve as an entry and exit point for this mosquito species, through human commerce. Apart from the favorable temperature at the coast, coastal mosquito population may be genetically similar to those from the Asian continent due to the frequent arrivals of shipping vessels or activities between these regions that could provide opportunities for introduction of new Asian *Ae. aegypti* populations (Moore *et al.,* 2013).

Whether the observed inherent difference in the competence to the virus between the *Aedes* populations from both sites is related to their genetics is unclear. It is known that *Aedes aegypti* exists in two forms or subspecies- *Aedes aegypti aegypti* and *Aedes aegypti formosus* (Scott *et al.,* 1975; Trpis *et al.,* 1978; Tabachnick *et al.,* 1979).

5.3 Vertical transmission of DENV among the Kilifi and Nairobi *Ae. aegypti* populations

The results demonstrated that there is occurrence of vertical transmission of DENV among Kilifi and Nairobi *Ae. aegypti* populations. Kilifi *Ae. aegypti* mosquito populations had a high dengue virus pooled infection rate compared to the Nairobi mosquito population. A high rate of TOT in the Nairobi *Ae. aegypti* population was observed at high temperature, similar to what was observed in the Nairobi *Ae. aegypti* infection rates. These observations therefore are consistent with observations made in a study done by Mourya *et al.,* (2001) that shows that susceptibility of *Ae.*

aegypti to dengue virus is associated with vertical transmission rates, since higher transovarial transmission in a strain could be due to the presence of a higher number of susceptible, infected females. The TOT results obtained from the present study is consistent with the studies done by Joshi *et al.* (2002), Wasinpiyamongkol *et al.* (2003), and Rohani *et al.* (2008). These studies demonstrated that the dengue viruses can undergo TOT over several generations of *Aedes aegypti* mosquitoes under laboratory conditions.

A study done by Thongrungkiat *et al.* (2011) also found that vertical transmission increase after several weeks of TOT-egg incubation at room temperature. Therefore, it was possible that these events might occur in nature, and could have important epidemiological consequences for dengue transmission. Many surveillance studies of natural TOT dengue virus have been performed. The findings from the present study were consistent with those of other studies that showed the occurrence of TOT for dengue virus. This is the first report on the occurrence of TOT of dengue virus by *Ae. aegypti* in Kilifi and Nairobi counties in Kenya.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The findings of this study demonstrated that:

- *Aedes aegypti* mosquito populations from Nairobi and Kilifi are susceptible to dengue-2 virus, with only Kilifi *Ae. aegypti* population supporting disseminated infection and transmission.
- Environmental temperature has a significant effect on the vector competence.

- It was observed that *Ae. aegypti* mosquitoes are susceptible to dengue-2 virus, and infected mosquitoes are able to vertically transmit the virus to their progeny. This is the likely mechanism by which the virus is maintained and propagated among its natural hosts.

6.2 Recommendations

In view of the current findings, there is need:

- To determine the susceptibility of *Aedes aegypti* to other serotypes of dengue virus (1, 3, 4) in the Kenyan *Ae. aegypti* mosquito populations.

- To improve knowledge of dengue transmission and establish a stringent surveillance system for Kenya and neighboring countries. This may help to identify the presence of the virus before the rapid spread, improve dengue prevention and control strategies.

- To examine the TOT indicator further with larger areas and over longer times, also taking into account various factors that could influence the TOT mechanism, such as rainfall, humidity, and temperature. This will provide an early warning signal of an impending dengue epidemic so that pre-epidemic control interventions can be implemented to suppress disease transmission and to prevent the disease spreading to new areas.

6.3 Study limitations

This study only covered three villages with almost similar features in terms of being closer to the natural forest in both Kilifi and Nairobi Counties. Consideration of more sites within the Counties was not possible because of minimal finances and limited time. This study did not distinguish between *Ae. aegypti aegypti* and *Ae. aegypti formosus.*

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Appendix 1: Kenya Medical Research Institute Scientific Streering 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

ESACIPAC/SSC/100630 **7th August, 2012**

Edith Chepkorir

Thro'
Director, CVR
NAIROBI

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

REF: SSC No. 2349 (Revised) – Determination of the effect of rearing temperature on the virus competence of *Ae. Aegypti* populations from Kilifi and Nairobi Counties for dengue virus.

Thank you for your letter dated 3rd August, 2012 responding to the comments raised by the KEMRI SSC.

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

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

Sammy Njenga, PhD
SECRETARY, SSC

CENTRE FOR VIRUS RESEARCH
10 AUG 2012
P. O. Box 54628 - NAIROBI

In Search of Better Health

Appendix 2: Kenya Medical Research Institute Ethical Review 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 **September 26, 2012**

TO: **Ms. EDITH CHEPKORIR (PRINCIPAL INVESTIGATOR)**

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

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

Dear Madam,

RE: **SSC PROTOCOL No. 2349 – 2ND REVISION (*RE-SUBMISSION*): DETERMINATION OF THE EFFECT OF REARING TEMPERATURE ON THE VECTOR COMPETENCE OF *AE. AEGYPTI* POPULATIONS FROM KILIFI AND NAIROBI COUNTIES FOR DENGUE VIRUS (*VERSION DATED 21ST SEPTEMBER 2012*)**

Reference is made to your letter dated September 21, 2012. The ERC Secretariat acknowledges receipt of the revised proposal on September 21, 2012.

This is to inform you that the Committee determines that the issues raised at the 207th ERC meeting of 5th September 2012 are adequately addressed. Consequently, the study is granted approval for implementation effective this **26th day of September 2012** for a period of one year.

Please note that authorization to conduct this study will automatically expire on **September 25, 2013**. If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to the ERC Secretariat by **August 14, 2013**. The regulations require continuing review even though the research activity may not have begun until sometime after the ERC approval.

You are required to submit any proposed changes to this study to the SSC and ERC for review and the changes should not be initiated until written approval from the ERC is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of the ERC and you should advise the ERC when the study is completed or discontinued.

Work on this project may begin.

Sincerely,

DR. CHRISTINE WASUNNA,
ACTING SECRETARY,
KEMRI ETHICS REVIEW COMMITTEE

CENTRE FOR VIRUS RESEARCH
27 SEP 2012
P.O. Box 54628 - NAIROBI

Appendix 3: Board of Postgraduate approval of research proposal letter

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: 254-067-52711/52181-4
FAX: 254-067-52164/52030

REF : JKU/BPS/TM305-1071/2011 **29TH January 2014**

Ms. Edith Chepkorir
C/o COHES
JKUAT

Dear Ms. Chepkorir,

RE: APPROVAL OF RESEARCH PROPOSAL AND SUPERVISORS

Kindly note that your research proposal entitled: **"Determination of the effect of rearing temparataure on the vector competence of Ae aegypti populations from Kilifi and Nairobi counties for dengue virus ".** The following are your approved supervisors:-

1. Prof. Zipporah Ng'ang'a
2. Dr. Rosemary Sang

Yours sincerely

PROF. GRACE N. NJOROGE
DIRECTOR, BOARD OF POSTGRADUATE STUDIES

Copy to: Principal, COHES
/pnk

Appendix 4: Publication

Chepkorir et al. Parasites & Vectors 2014, 7:435
http://www.parasitesandvectors.com/content/7/1/435

Parasites & Vectors

RESEARCH Open Access

Vector competence of *Aedes aegypti* populations from Kilifi and Nairobi for dengue 2 virus and the influence of temperature

Edith Chepkorir[1,3*], Joel Lutomiah[2], James Mutisya[2], Francis Mulwa[1], Konongoi Limbaso[2], Benedict Orindi[1], Zipporah Ng'ang'a[3] and Rosemary Sang[1,2]

Abstract

Background: Susceptibility of *Ae. aegypti* mosquito to dengue virus (DENV) varies geographically and can be influenced by climatic factors such as temperature, which affect the incidence, seasonality and distribution of vector-borne diseases. The first outbreak of dengue fever (DF) in Kenya occured in 1982 in the coastal towns of Malindi and Kilifi. Unlike Nairobi where no active dengue transmission has been reported, DF is currently re-emerging at the Coast causing major outbreaks. This study investigated the vector competence of *Ae. aegypti* populations from two urban areas, Kilifi (Coast of Kenya) and Nairobi (Central Kenya), for DEN-2 virus and the influence of temperature on the same.

Methods: Four-day old adult female *Ae. aegypti* mosquitoes collected as eggs from the two sites were exposed to defibrinated sheep blood mixed with DEN-2 virus ($10^{5.08}$ PFU/ml) using a membrane feeder. Half of the exposed mosquitoes were incubated at high temperature (30°C) and the other half at low temperature (26°C), and every 7 days up to day 21 post-infection 30% of the exposed mosquitoes were randomly picked, individually dissected, separated into abdomen and legs, and tested for midgut and disseminated infection, respectively, including virus quantification by plaque assay using Vero cells.

Results: Nairobi mosquito populations exhibited significantly higher midgut infection rates (16.8%) compared to the Kilifi population (9%; $p = 0.0001$). Midgut infection rates among the populations varied with temperature levels with a significantly higher infection rate observed for Nairobi at high (21.3%) compared to low temperature (12.0%; $p = 0.0037$). Similarly, for the Kilifi population, a significantly higher infection rate was recorded at high (11.6%) relative to low temperature (6.8%; $p = 0.0162$). It is however, noteworthy that disseminated infection was higher among the Kilifi mosquito population (40.7%) than in Nairobi mosquitoes (10.3%; $p < 0.0001$).

Conclusion: The findings show a clear inherent difference between the two populations in their ability to develop disseminated infection with high temperature having an added effect of enhancing vector competence. Therefore, the inherent difference among the two populations of *Ae. aegypti* coupled with prevailing ambient temperature could partly explain the distribution of dengue 2 virus between the Coastal and Nairobi regions in Kenya.

Keywords: *Aedes aegypti*, Vector competence, Temperature

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Background

Dengue virus, a mosquito-borne virus belonging to the genus *Flavivirus* and family *Flaviviridae,* exists in 4 distinct serotypes (DEN 1-4) [1]. DENV constitutes a major public health concern, infecting millions of people per year in tropical and subtropical areas globally [2]. Dengue illness in humans presents with a wide spectrum of clinical manifestations, ranging from a flu-like Dengue Fever (DF), to the more severe Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS).

Dengue fever caused by all four serotypes has been on the increase since 1980, mostly affecting Asia, South America and the Caribbeans [3,4]. Although there is limited documentation on the burden of Dengue in Africa [4] recent events suggest major re-emegence of the disease in Africa with outbreaks having been reported in East Africa (i.e South Sudan, Somalia, Kenya and Tanzania) [5] and West Africa (i.e Senegal, Central Africa Republic) [6]. In Kenya, the first outbreak of dengue fever caused by dengue virus 2 (DEN-2) occured in 1982 in the coastal towns of Malindi and Kilifi [7]. A serosurvey carried out in 2005 revealed the occurrence of dengue transmission in coastal and inland parts of Kenya [8]. In 2012, dengue outbreak was reported in Northern Kenya and subsequently in the coastal town of Mombasa (KEMRI lab reports 2012-2014) and is now viewed as a re-emerging public health problem in Kenya.

Aedes aegypti mosquito, the principal vector of DENV vector, originated in Africa and spread to tropical countries in the 17th and 18th centuries [9]. Urbanization is a major factor in facilitating the increase of *Aedes* mosquito species populations [1]. Accumulation of non-biodegradable, man-made containers used to store water in and around living areas provides the aquatic breeding environment required by these mosquitoes [10]. Based on these demographic changes and subsequent increases in *Aedes* mosquito populations, Appawu [11] predicted an increase in DENV transmission in Africa. It has been suggested that susceptibility of different strains of *Aedes* species and populations to DENV varies geographically affecting DENV distribution in Africa [12] possibly driven by inherent and climatic factors [13,14].

Vector competence (VC) is the intrinsic permissiveness of an arthropod vector for infection, replication and transmission of a virus [15], mediated by the presence of several genetically determined barriers to viral transmission, including a midgut infection barrier (MIB) that prevents invasion and replication of the viruses and a midgut escape barrier (MEB) that prevents dissemination to other tissues [16]. These barriers are major determinants of vector competence to DENV during experimental infections [17]. The barriers also vary in prevalence in natural populations, leading to large intraspecific variation of *Ae. aegypti* vector competence and may determine the epidemiology of dengue viruses [16].

Ae. aegypti is widely distributed across Kenya [18] and the risk of dengue virus transmission is therefore likely to be equally widespread. Dengue outbreaks have been reported multiple times at the Kenyan coastal towns of Malindi, Kilifi [7] and most recently Mombasa (KEMRI lab reports 2012-2014), but in spite of the presence of *Ae. aegypti* in Nairobi DENV transmission has not been documented. Whether these outbreaks are related to the urbanization and the area being a tourist destination is unclear. Like the coastal towns, Nairobi the capital city of Kenya is a major international hub hosting the largest airport in the East African region, facilitating the influx of a large number of international travelers. To explain the differences in outbreak occurrence between Nairobi and the Coast, we hypothesized that the vectorial capacity of the *Ae. aegypti* populations between the two areas are different. While inherent differences between *Ae. aegypti* populations may exist, the contribution of differential ambient temperatures between these sites in driving the observed DEN transmission patterns has never been assessed. Therefore, this study sought to evaluate the vector competence of *Ae. aegypti* populations from Nairobi and Kilifi in laboratory experiments while incorporating the effect of prevailing ambient temperatures at these sites.

Methods

Ethical considerations

Scientific and ethical approval was obtained from the Kenya National Ethical Review Committee at the Kenya Medical Research Institute (protocol KEMRI/RES/7/3/1) dated 26th September, 2012. Animal use approval was obtained from the KEMRI Animal Care and Use Committee (ACUC).

Study area

The study sites included the Coastal and Central parts of Kenya (Figure 1). At the coast, eggs of *Ae. aegypti* mosquitoes were collected from villages in Rabai in Kilifi County based on prior history of dengue circulation in the area [7]. Kilifi County (latitude of 3.63°S and a longitude of 39.85°E) has a mean daily temperature of 30°C, rainfall of approximately 88.25 mm and 82% relative humidity. Collections in Nairobi were from Karura on the outskirts of Nairobi (latitude of 1.28°S and a longitude of 36.81°E) with a mean daily temperature of 25°C, rainfall of approximately 85.35 mm and 70% relative humidity. The study adopted a laboratory-based experimental design.

Mosquito egg collection

Aedes aegypti eggs were collected using oviposition cups (ovicups), lined with oviposition papers and half-filled with water. These were set at various points, at least 100 meters from each other, in the study area for 4 days with the Global Positioning System (GPS) coordinates of each

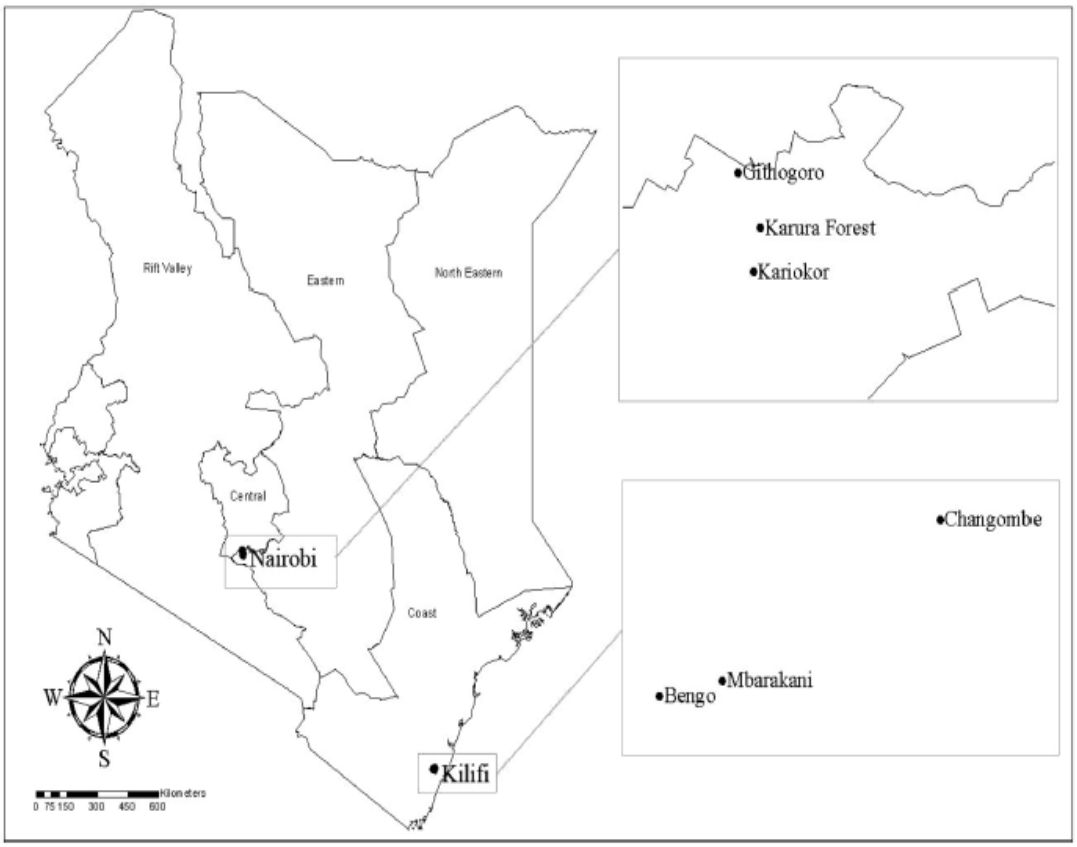

Figure 1 Map showing the study area and the sites where *Ae. aegypti* eggs were collected.

ovicup point taken for geo-referencing. On day 4, all the ovicups were collected and the eggs transported to the biosafety level-2 (BSL-2) insectary at KEMRI in Nairobi, where they were dried on damp cotton wool to quiescent state [19], and stored in an air tight container at room temperature in the insectary.

Mosquito rearing

Mosquitoes were reared in the KEMRI insectary, maintained at a temperature of 28°C and 80% relative humidity (RH), with a 12:12-hour (Light:Dark) photoperiod. As needed, several batches of eggs (F_0) from Kilifi and Nairobi areas were dispensed in water on larval trays for hatching in a level 2 insectary. The larvae were fed on yeast mixed with tetramin fish food until they pupated. The pupae were collected every morning and put in a cup containing water. The cup with pupae was placed in a 1-gallon plastic cage with a netting material on top and allowed to develop into F_0 adult mosquitoes. The emerging adults were knocked down by placing them in a -20°C freezer for one minute, then morphologically identified under a dissecting microcope using taxonomic keys of Edwards [20], to ensure that only *Ae. aegypti* mosquitoes were used in the subsequent experiments. Positively identified *Ae. aegypti* mosquitoes were returned to their experimental cages, blood fed on clean laboratory-bred mice and provided with oviposition papers to lay F_1 eggs. The F_1 eggs were hatched and reared as decribed above. Only adult female mosquitoes were used in the subsequent experiments [21].

Dengue virus amplification

Dengue virus serotype 2 (DEN-2) which was isolated from a patient's sample (Sample number: 008/01/2012) from the 2012 dengue outbreak in Mandera, Kenya, was used in the study. The virus was passaged in T-75 cm^2 culture flask containing C6/36 cell lines (*Ae. albopictus* mosquito cell lines), grown in Dulbecco's modified Eagle's medium (DMEM), (GIBCO® Invitrogen corporation, Carlsbad, California), liquid (4.5 g/L D-glucose) without L-glutamine and sodium pyruvate, supplemented with 10% heat-inactivated fetal bovine serum (FBS), (Sigma-Aldrich, St. Louis, MO), 2% L-Glutamine (Sigma-Aldrich, St. Louis, MO), and 2% antibiotic/

antimycotic solution with 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml (Sigma-Aldrich, St. Louis, MO) and incubated at 28°C in 5% CO_2 over night. Confluent monolayer of C6/36 cells were inoculated with 600 μl of the dengue virus supernatant isolate and incubated for 1 hour with frequent agitation/rocking to allow for virus adsorption. The infected cells were maintained in DMEM supplemented with 2% FBS, 2% L-Glutamine and 2% antibiotic/antimycotic, incubated at 28°C in 5% CO_2 and observed daily for cytopathic effect (CPE) for a period of 7 days. Once the CPE was observed to affect 80% of the monolayer, the flask was frozen overnight at -80°C, thawed on wet ice, then clarified by centrifugation at 3000 revolutions per minute for 10 minutes and the supernatant harvested by aliquoting into 1.5 ml cryovials. All the aliquots were stored at -80°C [22].

Dengue virus quantification

Quantification of dengue virus was performed by plaque assay. Ten fold serial dilutions of the amplified DENV was carried out and inoculated in 6 well plates containing confluent Vero monolayers as described by Gargan *et al.,* [23]. This was grown in Minimum Essential Medium Eagle (MEM), (Sigma-Aldrich, St. Louis, MO) with Earle's salts and reduced $NaHCO_3$, supplemented with 10% heat-inactivated fetal bovine serum (FBS), (Sigma-Aldrich, St. Louis, MO), 2% L-Glutamine (Sigma-Aldrich, St. Louis, MO), and 2% antibiotic/antimycotic solution with 10,000 units penicillin, 10 mg streptomycin and 25 μg amphotericin B per ml (Sigma-Aldrich, St. Louis, MO) and incubated at 37°C in 5% CO_2 over night. 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 9 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 [23]:

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

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

Susceptibility studies

Mosquito infection

An infectious blood meal was prepared by mixing DEN-2 virus stock with a virus titer of $10^{5.08}$ plaque-forming units (PFU)/ml and defibrinated sheep blood, in a ratio of 1:1, to end up with $10^{3.03}$ PFU/ml virus concentration. The virus/blood mixture was put in membrane feeders covered with a freshly prepared mouse skin [24], and maintained using the Hemotek system which employs an electric heating element to maintain the temperature of the blood meal at 35°C ± 1°C [25]. Four-day-old adult female mosquitoes were allowed to feed on the infectious blood meal through the mouse skin for 1 hour. After feeding, fully engorged mosquitoes were selected and put in secured cages, where they were maintained on 8% glucose for 7 to 21 days at set temperatures and 80% relative humidity. The first set of experiments involved maintaining both Kilifi and Nairobi exposed mosquitoes to temperatures set at an average of 26°C (i.e. similar to Nairobi temperature conditions). The second set of experiments involved maintaining another group of exposed mosquitoes from both sites to temperatures set at an average of 30°C (i.e. similar to Kilifi temperature conditions). Mortality was monitored in the cages by removing and counting dead mosquitoes daily [25].

Test for infection and dissemination rates of Dengue-2 virus

After every 7 days up to day 21 of incubation, 30% of live exposed mosquitoes were randomly picked and each dissected to separate the abdomens and legs. Individual abdomens and legs were placed separately in 1.5 ml eppendorf tubes containing 500 μl of homogenizing media (HM), consisting of MEM, supplemented with 15% FBS, 2% L-Glutamine, and 2% antibiotic/ antimycotic. The individual abdomens were homogenised using plastic grinders and the supernatant diluted in 10 fold serial dilutions. The dilutions were inoculated in confluent Vero cell lines in 12-well plates, grown in MEM, supplemented with 10% FBS, 2% L-Glutamine and 2% antibiotic/antimycotic. The infected cell monolayers were then overlaid with methylcellulose supplemented with 2% FBS, 2% L-Glutamine and 2% antibiotic/ antimycotic and incubated at 37°C in 5% CO_2. On day 9, 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. For each positive abdomen, corresponding legs were homogenised and their infection status determined as described above for the abdomens. Plaques were counted and calculated to determine the viral titer [26].

Data analysis

Analyses were performed using R version 2.15.1 [27]. To gain some insight into the dataset, descriptive statistics and graphical displays were used. Data on *Ae. aegypti* mosquito infection and dissemination rates at the two temperature levels and/or sites were compared using Chi square (and Fisher's exact) test at 5% significance

level and confidence intervals (CIs) for the proportions estimated [28,29].

Results

Susceptibility to dengue- 2 virus infection in Nairobi and Kilifi mosquitoes

Dengue 2 virus infection rates were measured in a total of 517 *Ae. aegypti* female mosquitoes in three replicates from Nairobi region (249 in low and 268 in high temperature regimes). Of these, 87 mosquitoes (16.8%; 95% CI: 13.7-20.20.3%) had DEN-2 infection in the midgut. The proportion of Nairobi mosquitoes with midgut infection was significantly higher in high temperature (21.3%) than at low temperature (12.0%; $p = 0.0037$). Figure 2A presents the percentage of infected mosquitoes at day 7, 14 and 21 post-infection for each temperature level with the highest infection recorded on day 14 post-infection at a high temperature of 30°C.

Kilifi mosquito population infection rates were measured in a total of 600 *Ae. aegypti* female mosquitoes in three replicates (300 in low and 300 in high temperature regimes). Of these, 54 mosquitoes (9%; 95% CI: 6.8-11.6%) developed midgut infection, with a significantly higher proportion recorded at high temperature (11.6%) relative to low temperature (6.8%; $p = 0.0162$). The percent of infected mosquitoes at day 7, 14 and 21 post-infection for each temperature level is presented in Figure 2B. There were low infection rates at 14 days post-infection under high temperature, with the highest infection at day 21 for both temperature levels.

Overall data for both Nairobi and Kilifi mosquito populations showed that 141 of the 1117 mosquitoes (12.62%; 95% CI: 10.73-14.71%) had midgut infection with a significantly higher proportion recorded in Nairobi (16.8%) than Kilifi (9%; $\chi^2 = 14.73$, $df = 1$, $p = 0.0001$). Analysis from both experiments showed that mosquito infection was significantly influenced by temperature with higher infections recorded for mosquito populations from Nairobi relative to Kilifi at both temperature levels (Figure 3).

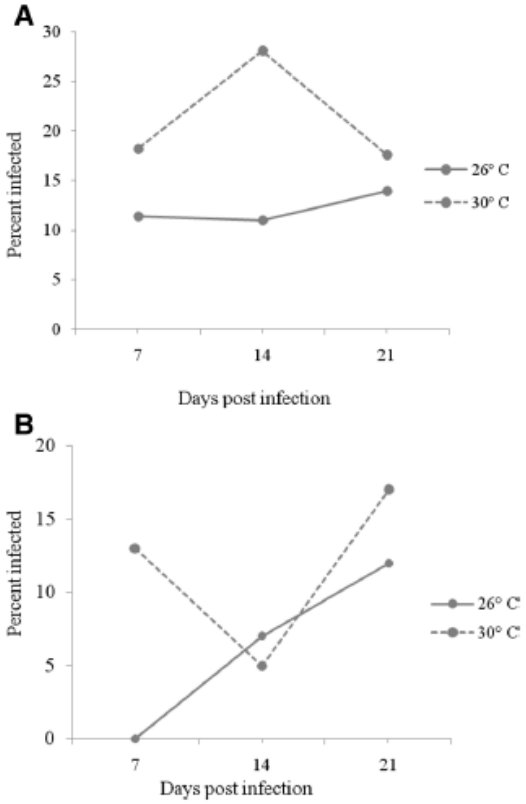

Figure 2 Proportion of Nairobi (A) and Kilifi (B) *Ae. aegypti* infected at day 7, 14 and 21 post-infection at each temperature level.

Dissemination of dengue-2 virus in Nairobi and Kilifi mosquitoes

The proportion of mosquitoes with disseminated infection was significantly higher for Kilifi mosquito population (40.7%; 95% CI: 27.6-55.0%) relative to that of Nairobi population (10.3%; 95% CI: 4.8-18.7%; $p < 0.0001$). This was evident at both the low and high temperature levels (Figure 4). Figure 4 also shows that, while the highest dissemination was observed for Kilifi population at the high temperatures, the lowest was recorded for Nairobi mosquito population, a finding which contrasts the pattern observed with midgut infection rates, in which the highest rates were observed in the Nairobi mosquitoes.

The results further showed that, for the Nairobi mosquito population, a higher disseminated infection was recorded at low (16.7%, $n = 30$) relative to high temperature (7.02%, $n = 57$), although the difference was not significant ($p = 0.2648$). Our findings therefore, revealed a negative association between temperature and the dissemination of DEN-2 virus by the Nairobi mosquito population. In

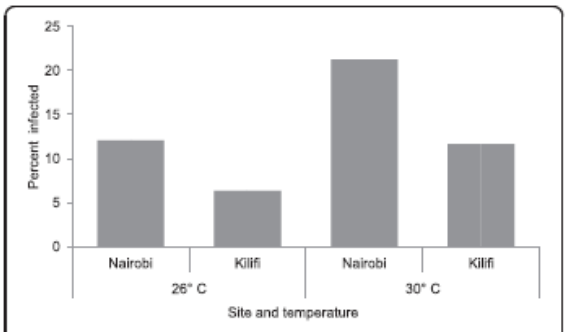

Figure 3 Percent mosquitoes infected per site at each temperature level.

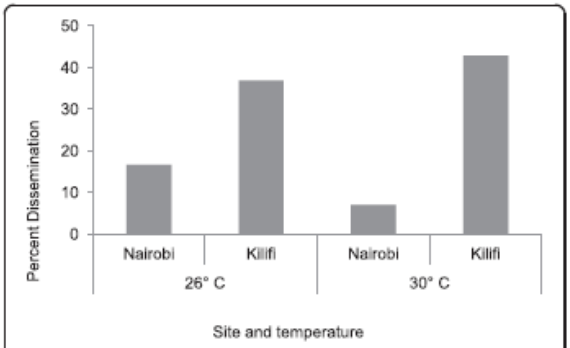

Figure 4 Percent of infected mosquitoes with disseminated infection per site at each temperature level.

contrast for Kilifi mosquito population, a lower dissemination rate was recorded at low (36.84%) compared to at high temperature (42.86%), although the difference was not significant ($\chi^2 = 0.0195$, df = 1, p = 0.889).

Discussion

There are a number of factors that contribute to the vectorial capacity of a mosquito for an arbovirus, including mosquito survival, density, proportion of infected mosquitoes that are feeding, length of extrinsic incubation period, vector susceptibility to the virus, and density of susceptible hosts [30]. We investigated *Ae. aegypti* vector susceptibility to midgut infection and ability to disseminate dengue-2 virus, as a measure of the extent of adaptation of a virus to the vector and its potential to transmit it. However, the full competence of a vector is established by both its ability to become infected and to transmit the virus [26]. This parameter gives vector competence its epidemiologic importance. In this study, transmission potential was estimated from dissemination rates because previous studies have suggested that mosquitoes are capable of transmitting DENV as long as the virus is able to disseminate from the midgut into the hemocoel, subsequently finding its way to the salivary glands for transmission [31,32]. An assumption was made based on these previous studies that mosquitoes that have a disseminated infection were capable of transmitting.

This study constitutes the first initial effort to understand the role of *Ae. aegypti* in driving dengue transmission in Kenya, to assess the risk of further spread of the disease by analyzing the vector competence of *Ae. aegypti* populations from parts of Kenya and the influence of environmental factors such as temperature, that prevail in outbreak hotspots and other areas of potential risk. The overall findings from this study demonstrate that the Nairobi *Ae. aegypti* population is a relatively inefficient vector for DEN-2 virus compared to that from Kilifi with the Nairobi population depicting high infection, but low dissemination rates in both low and high temperature settings. These findings suggest a weak midgut infection barrier (MIB) and a strong midgut escape barrier (MEB) for the Nairobi population and a moderate MIB, but weak MEB for the Kilifi population. The Kilifi *Ae. aegypti* population demonstrated inherent capacity to be more efficient in Dengue-2 virus transmission than the Nairobi population. Overall, the vector competence is considered as being inversely proportional to the level of MIB and MEB in the mosquito population: strong MIBs and MEBs reduce the potential for infection and dissemination and eventually transmission by the vector [17].

Geographic variation in *Ae. aegypti* populations to DENV susceptibility has been reported in various studies [31,33]. A study done by Moncayo [30] on populations from various geographical locations showed that *Ae. aegypti* from Galveston, Texas, were more susceptible than those from Bolivia, but were less susceptible than mosquitoes from Thailand. Similar observations were made by Bennett [17] on *Ae. aegypti* collections from various locations in Mexico which differed significantly in their midgut susceptibility to infection. Our findings are similar to these studies, because the midgut and disseminated infection rates differed significantly with *Ae. aegypti* populations collected from the two different sites, even after exposure to the same conditions in the laboratory.

Studies have also shown that temperature is one of the most important factors affecting biological processes of mosquitoes including their interaction with viruses [34,35]. Our results demonstrate a significantly higher infection rate at high temperatures for mosquitoes from both Nairobi and Kilifi, which is consistent with results by Watts [34]. Although disseminated infection for many mosquito-borne viruses is known to be affected by temperature, this was not clearly observed in this study, but transmission of an arthropod-borne pathogen is only possible where there is disseminated infection [32].

Aedes aegypti is reported to have originated in Africa, adapted to the peridomestic environment of African villages before being exported to America by the slave trade and elsewhere by commercial transportation [36]. According to studies carried out by Moore [37], populations of *Ae. aegypti* outside Africa consist of mosquitoes arising from one of two ancestral clades. One clade is basal and is primarily associated with West Africa while the second arises from the first and contains primarily mosquitoes from East Africa. Mombasa at the Kenyan Coast is the second largest city of Kenya and a major port. It could therefore serve as an entry and exit point for this mosquito species, through human commerce. Apart from the favorable temperature at the coast, coastal mosquito populations may be genetically similar to those from the Asian continent due to the frequent

arrivals of shipping vessels or activities between these regions that could provide opportunities for introduction of new Asian *Ae. aegypti* populations [37].

Whether the observed inherent difference in the competence to the virus between the *Aedes* populations from both sites is related to their genetics is unclear. It is known that *Aedes aegypti* exists in two forms or subspecies- *Aedes aegypti aegypti* and *Aedes aegypti formosus* [38-40]. While acknowledging the taxonomic difficulty in distinguishing these forms apart based on morphology [41], it may be worthwhile to understand in future studies how the populations between the sites compare genetically.

Environmental conditions play a major role in infection of viruses. High environmental temperature increases virus multiplication to high titers and reduces the extrinsic incubation period for the virus to be established within the vector [35]; get to the salivary glands after which it can be transmitted to another host [34]. Relative humidity on the other hand enhances mosquito survival allowing it to survive long enough for virus extrinsic incubation period to be complete allowing for virus transmission. Kilifi County experiences high annual temperature, rainfall and relative humidity as compared to Nairobi County which could explain why there have been several dengue fever outbreaks in the region. Data presented here reveals that the infection rates of DEN-2 virus in *Ae. aegypti* were significantly higher at high temperature regimes for both mosquito populations, suggesting a potentially significant role of temperature in the dynamics of DENV transmission. Both Kilifi and Nairobi *Ae. aegypti* populations were incubated at conditions that mimic the environmental conditions of both sites, but still low disseminated infection was observed in the Nairobi population. Like several earlier studies [34,35], our findings highlight the need for a better mechanistic understanding of the environmental determinants of vector pathogen interactions.

Conclusions

The findings show that both mosquito populations are susceptible to dengue-2 virus, with only Kilifi population supporting disseminated infection and transmission. The findings suggest an inefficient transmission ability of DEN-2 virus by the Nairobi *Ae. aegypti* population. This could explain why there has been no evidence of active dengue transmission in Nairobi despite reported cases of dengue in health facilities usually from individuals who have travelled from Mombasa (coast) and/or Mandera (North-eastern Kenya) where outbreaks have been reported. Environmental temperature also has a significant effect on the vector competence as evidenced from our results, which further explains ready transmission of dengue in Mombasa where mean temperatures are higher than Nairobi. Variation in vector competence among the populations of *Ae. aegypti* examined may help explain the distribution and spread of dengue fever. As the impact of climate change leads to increasing temperatures, spread of the virus through local vectors into susceptible host populations becomes likely. One of the limitations of this study is that it did not distinguish between *Ae. aegypti aegypti* and *Ae. aegypti formosus*. Therefore, it would be worthwhile for future studies to investigate how the two populations of this vector compare genetically in order to shed light on the observed inherent differences between the two populations in transmitting dengue-2 virus.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EC contributed in mosquito eggs collection, rearing, vector competence studies, data analysis, drafted manuscript, and final manuscript preparation. JL contributed to field study design, vector competence studies, drafting manuscript, and final manuscript preparation. JM and FM contributed to mosquito egg collection, mosquito rearing, identification and review of the manuscript. KL contributed to drafting manuscript, and final manuscript preparation. BO contributed to data analysis and final manuscript preparation. RS and ZN contributed to overall study design, overall supervision of implementation, data analysis, and final manuscript preparation. All authors read and approved the final manuscript.

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