

**DENGUE 2 INFECTION IN WILD CAUGHT *Papio anubis* (OLIVE
BABOONS) AND *Chlorocebus aethiops* (AFRICAN GREEN
MONKEY) FROM SELECTED REGIONS OF KENYA**

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P150\21571\2010

**A Thesis Submitted in Partial Fulfillment of the Requirements for the
Award of the Degree of Master of Science in Infectious Diseases
(Molecular Virology) in The School of Health Sciences of Kenyatta
University.**

NOVEMBER 2014



Declaration

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

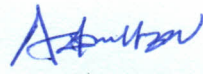

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Dedication

This thesis is dedicated to my mother Caroline Ambala who laid foundation to my education, Andrew Ayany who has been encouraging me to accomplish this work and not forgetting my sisters Everlyne Akinyi, Hazel Anyango, Jacqueline Achieng and Vanessa Adhiambo for their support during the entire duration of my studies. You have always been a source of my inspiration.

Acknowledgement

I wish to thank my supervisors, Dr. Jeneby Maamun, Institute of Primate Research who gave me invaluable intellectual advice, Dr. Anthony Kebira, Kenyatta University for accepting to supervise my work as his student.

I am grateful to the Institute of Primate Research for the support accorded in the molecular and virology laboratories during my studies.

I also wish to acknowledge Mr. Shem Muturi, Mr. Damian Odoyo, Mr. Elephas Munene (virology laboratory) and Mr. Samson Mutura (molecular laboratory) for continued technical assistance.

I also recognize the companionship of my fellow student colleagues at Institute of Primate Research. I would finally like to thank all those who gave me personal encouragement and assisted me in many ways.

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Abbreviations

1X	One times the normal concentration
<i>A</i>	Aedes
<i>Ae</i>	Aedes
AC	Alternating Current
AGM	African green monkey
C	Capsid
<i>C. aethiops</i>	<i>Chlorocebus aethiops</i>
cDNA	Copy deoxyribonucleic acid
CO ₂	Carbon dioxide
DC	Dendritic cell
DC-SIGN	Dendritic cell specific intracellular adhesion molecules grabbing intergrin
DENV	Dengue virus
DENV 1	Dengue virus 1
DENV 1-4	Dengue virus 1-4
DENV 2	Dengue virus 2
DENV 4	Dengue virus 4
DHF	Dengue hemorrhagic fever
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic Reticulum
GAGs	Glycosaminoglycans
ICAM	Intercellular adhesive molecule

IgG	Immunoglobulin gamma
IgM	Immunoglobulin M
NHPs	Non-human primates
nm	Nanometer
NS	Non-structural proteins
OD	Optical density
OPD	Orthophenylenediamine
<i>P. anubis</i>	<i>Papio anubis</i>
PBS	Phosphate buffered saline
PBS\Tween	PBS containing Tween 20
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
Rpm	Rotations per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
TAE	Tris acetate EDTA buffer
TS2	Type specific primer to dengue 2
WHO	World Health Organization
EtBr	Ethidium Bromide

Abstract

Dengue viruses, family Flaviviridae and genus Flavivirus occur as four antigenically distinct serotypes denoted as dengue virus 1, 2, 3, 4 (DENV-1, 2, 3, 4). The four serotypes are known to broadly infect different vertebrate hosts including humans, wild animals and various species of non-human primates (NHPs). Despite the fact that NHPs have increasingly been implicated as potential sources of emerging zoonotic viral diseases, there is no available scientific data on sylvatic dengue virus surveillance in Kenya. The present study investigated the sero-prevalence of DENV-2 exposure and characterized DENV serotypes circulating in NHPs from Laikipia, Aberdares, Kajiado, Kibwezi, Kitale, Nairobi and Namanga regions. A total of 287 wild-caught NHPs, comprising of 115 Olive baboons (*Papio anubis*) and 172 Africa Green monkey-AGM (*Chlorocebus aethiops*) were sampled. Sera prepared from the 287 blood samples were tested for anti-dengue viral immunoglobulin gamma (IgG) antibody using enzyme linked immunosorbent assay (ELISA). Samples which were positive for anti-dengue IgG were subsequently subjected to a nested polymerase chain reaction (PCR) test with specific primers targeting the capsid and pre-membrane genes of the virus. The sero-prevalence of dengue virus exposure was 35.7% (41/115) in *P. anubis* and 39.5% (68/172) *C. aethiops*. RT-PCR confirmed that 1.7% (2/115) *P. anubis* and 2.3% (4/172) *C. aethiops* were positive for dengue virus. The 2 baboons and 4 AGMs were from Laikipia, Kakamega, Kibwezi and Kitale respectively. Chi-square test indicated no statistical significance in sero-prevalence ($P = 0.537$) results between the species. In conclusion, this study provides strong evidence of dengue virus infection among *P. anubis* and *C. aethiops* in Kenya. Although this epidemiological study indicates high dengue exposure in NHPs, there was significantly low active infection as evidenced by PCR results. My conclusion is that sylvatic dengue viruses 2 circulates in non-human primates found in Kenya. I recommend that the country should strengthen its surveillance on sylvatic dengue viruses which will likely become a serious public-health problem in the near future in human population that live in close proximity to animals.

CHAPTER ONE: INTRODUCTION

1.1 Introduction

Dengue virus (DENV) is an arbovirus (arthropod transmitted virus) belonging to the family Flaviviridae and genus flavivirus. Arboviruses such as Dengue viruses, Yellow fever virus and West Nile virus are important causes of human diseases worldwide. These arboviruses are known to circulate among various wild mammals with frequent spill over transmission to humans and domestic animals which are incidental or dead-end hosts (Weaver and Reisen, 2010). Although some of the DENV serotypes have lost the requirement for enzootic amplification, the sylvatic DENV-2 is capable of causing spill over to human population causing human dengue fever or even dengue hemorrhagic fever as reported in West Africa and Malaysia (Rudnick *et. al.*, 1986; Vasilakis *et. al.*, 2008a; Weaver and Reisen, 2010).

Dengue virus is a zoonotic infection infecting both humans and non-human primates (NHP). It circulates in two different cycles, the endemic cycle and the sylvatic cycle respectively. Sylvatic DENV strains are ecologically and evolutionary different from endemic strains (Wang *et. al.*, 2000). An analysis of three complete genomes obtained from human samples taken in 1966 from Nigeria , were found to be of West African sylvatic DENV-2 origin (Vasilakis *et. al.*, 2008). However, recent evidence based on complete genome phylogenetic analysis suggests that this strain falls into human dengue diversity of endemic DENV-1 (Vasilakis *et. al.*, 2008).

In 2005, a sylvatic DENV-1 was detected from a febrile patient in Malaysia clustered with the ancestral sylvatic DENV-1 which was isolated from a sentinel monkey in 1972 (Teoh *et. al.*, 2010). In the thirty-two samples that had DENV, 14 samples had DENV-2

from both man and NHP (Carey *et. al.*, 1971). Another case of sylvatic strain infecting humans was reported in France among travelers returning from Senegal and Mali, West Africa (Franco *et. al.*, 2011a). Genetic analysis of isolates from both sites revealed 99.6% sequence identity and phylogenetic analysis revealed that all the sequences were clustered with West African sylvatic DENV-2 (Renaudat *et. al.*, 2009). The first documented case in Asia of dengue hemorrhagic fever (DHF) involving sylvatic DENV was observed in Malaysia (Cardosa *et. al.*, 2009). This Malaysian strain sequence clustered with the sequences of Asian sylvatic dengue strains that were recovered from monkeys in the 1970s (Cardosa *et. al.*, 2009). Although sylvatic DENV is associated with mild disease, Franco and others (2011a) have reported DHF caused by sylvatic DENV-2 of West African lineage. The findings suggested that sylvatic strains of DENV might have a greater pathogenic potential than previously thought (Vasilakis *et. al.*, 2010).

Sylvatic DENV -1, DENV-2 and DENV-4 have been isolated in Asia and West Africa (Wang *et. al.*, 2000), although the last isolation of a sylvatic dengue virus (DENV-4) occurred in 1975. Phylogenetic analysis suggests that sylvatic DENV are the ancestors of those viruses that now circulate endemically in human populations (Wang *et. al.*, 2000).

There is still a great potential for endemic forms of DENV to emerge from sylvatic cycles between mosquitoes and non-human primates (Vasilakis *et. al.*, 2007). This study proposed to detect and characterize DENV circulating in Olive Baboons (*Papio anubis*) and Africa Green monkeys (*Chlorocebus aethiops*) from selected geographical regions in Kenya.

1.2 Statement of Problem

Dengue infections afflict approximately 40% of the world population and approximately

cause more than 20,000 deaths per year. This disease is further complicated with lack of appropriate antiviral drugs and vaccines. In addition, misdiagnosis of this infection with diseases like malaria, leads to increased dengue fatality.

Even though primates are increasingly implicated as potential reservoirs and sources of emerging zoonotic diseases such as dengue hemorrhagic fever, there is no available data on dengue virus circulating in non-human primates found in Kenya and the transmission of sylvatic dengue 2 virus to humans. This study will immensely influence disease surveillance and human vaccine production.

1.3 Justification

Olive baboons and African green monkeys (AGM) are widely distributed non-human primates in Kenya. They are found in different ecological zones ranging from semi-arid regions, savanna, tropical rainforest and they live in close proximity to human populations in most parts of the country. However, as potential reservoirs of various zoonotic pathogens including DENV, information on sylvatic DENV in olive baboons and AGM in Kenya is still lacking. With the frequent reported dengue outbreaks in Kenya since 1982-2013, the surveillance of DENV in NHPs is of importance in providing information on reservoir hosts of the disease. Therefore this study was designed to detect and characterize DENVs circulating in Kenyan Olive baboons and AGMs.

This study will immensely influence disease surveillance and vaccine production.

1.4 Research Question

Does dengue virus sero-type 2 circulate among non-human primates, olive baboons (*Papio anubis*) and Africa Green monkey (*Chlorocebus aethiops*) found in different selected geographical regions in Kenya?

1.5 Hypothesis

Olive baboons and African green monkeys found in Kenya are not infected with enzootic dengue virus 2 strains.

1.6 Objectives

1.6.1 General Objective

To determine and molecularly characterise dengue virus 2 strain circulating in olive baboons and African green monkeys (AGM) from Laikipia, Aberdares, Kajiado, Kibwezi, Kitale, Nairobi and Namanga regions in Kenya.

1.6.2 Specific Objective

1. To detect and determine the sero-prevalence of dengue viral strains/serotypes in *Papio anubis* (Olive Baboons) and *Chlorocebus aethiops* (AGM) in Kenya.
2. To characterise dengue virus in *Papio Anubis* (olive baboons) and *Chlorocebus aethiops* (Africa Green monkey) populations using molecular methods.

CHAPTER TWO: LITERATURE REVIEW

2. 1 Dengue Virus

Dengue viruses (DENV) are among the most widely distributed arboviruses in tropical and sub-tropical regions in the world (Domingo *et. al.*, 2011). It is a positive sense, single stranded RNA enveloped virus of 50 nm in diameters. Dengue viral disease is caused by four DENV serotypes, DENV-1, 2, 3 and 4 (Wang *et. al.*, 2000). These viruses are transmitted to humans by mosquitoes of the genus *Aedes* (Gubler, 1998; Gubler *et. al.*, 2002). DENV virus circulates in humans (endemic cycles) and non-human primates (sylvatic cycles) mostly in forests. Serotype 1, 3 and 4 of DENV circulates in humans while serotype 2 circulates mainly in NHPs\ sylvatic mosquitoes. The endemic (Human\urban\peridomestic mosquitoes) cycle is principally vectored by *Aedes aegypti*. Other species that transmit endemic virus include *A. albopictus*, *A. polynesiensis* and *A. scutellaris*. The sylvatic (enzootic) cycle which involves NHPs is transmitted by species of *Aedes* mosquito such as *Ae. Furcifer* (Wang *et. al.*, 2000; Wolfe *et. al.*, 2001; Vasilakis *et. al.*, 2007).

Factors that influence the transmission of dengue virus can be divided into vector factors, human factors and environmental factors. The vector factors include: vector efficiency and virus infectivity (Amarasinghe, 2011). Human factors include sex, poverty, migration and race (de la C Sierra *et. al.*, 2007; da Silva-Nunes *et. al.*, 2008). To develop severe dengue hemorrhagic fever (DHF) factors like age, repeated dengue infections with a secondary serotype, genetic factors, virus genotype and nutritional status play a major role (Malavige *et. al.*, 2004; Wilder-Smith and Schwartz 2005; Rothman 2010; Stephens, 2010). Environmental factors include climate (Quintero *et. al.*, 2014).

2.1 Transmission Of Dengue Virus From Non-Human Primates To Humans

There is an enzootic dengue transmission cycle in the forest involving *Aedes* mosquitoes and lower primates in Africa and Asia but because there is rarely movement of the enzootic cycle into urban areas, the most important cycle is the urban transmission cycle. Because of high viremia resulting from dengue infection of humans, the viruses are efficiently transmitted between mosquitoes and humans without the need for an enzootic amplification host (Whitehead et al, 2007).

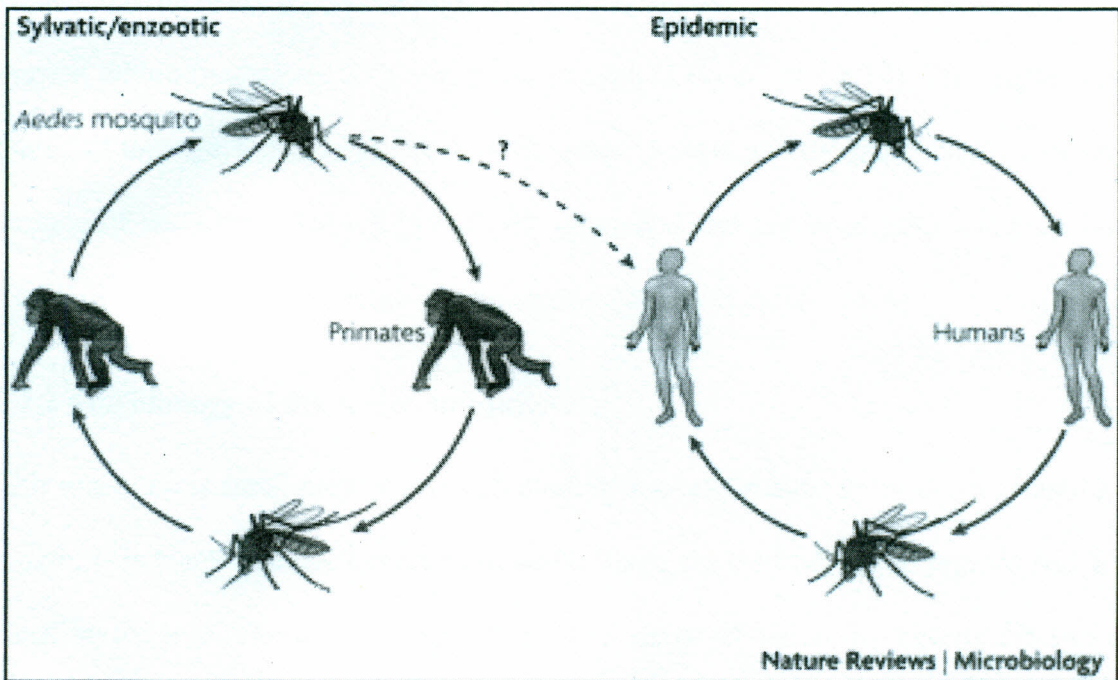


Fig. 2 :The Sylvatic and Urban Dengue Transmission Cycles (Whitehead *et. al.*, 2007)

2.2 The Vector: *Aedes aegyptia*

2.2.1 Life Cycle of *Aedes aegyptia*

The main vector of dengue is the female *Aedes* mosquito, which is also the principal vector of yellow fever. *Aedes* species mosquitoes require a blood meal for oviposition. These species have a complex life cycle. They undergo complete metamorphosis (Hopp and Foley, 2001).

Once a female mosquito takes a blood meal, it takes two or more days for the female to digest the blood, lay a batch of eggs, and seek another blood meal. This can be repeated many times in a female's lifetime. Only one mating is required to fertilize her lifetime egg production Fig. 2.1 (Levi *et. al.*, 2014).

Under optimal conditions, hatching of eggs of an *Aedes* mosquito occurs days to weeks when the water level rises into a larva. All four larval stages are aquatic. The mosquito larva molt four times in approximately a span of four days to complete their development which takes an average of 6-10 days. The larvae usually mature within two weeks to develop into a pupa (Levi *et. al.*, 2014). The pupae release the adult through a split in the back of the pupal cuticle and the pupal case floats on the surface of the water. The wings are fully expanded and hardened after about 24 hours. The mosquitoes live on average one or two months (Levi *et. al.*, 2014).

2.2.2 Morphology of the *Aedes* mosquito

This mosquito is small usually between three to four millimetres in length discounting leg length. It is black in color except from white 'spots' on the body, head regions and white rings on the legs. The thorax has a white 'Lyre' shape of which the 'chords' are two dull yellow lines. Its wings are translucent and bordered with scales. At rest, the insect turns up its hind legs in a curved fashion and usually cleans them by rubbing one against the other, or exercises them by crossing them and alternately raising and lowering them (Roland, 1998).

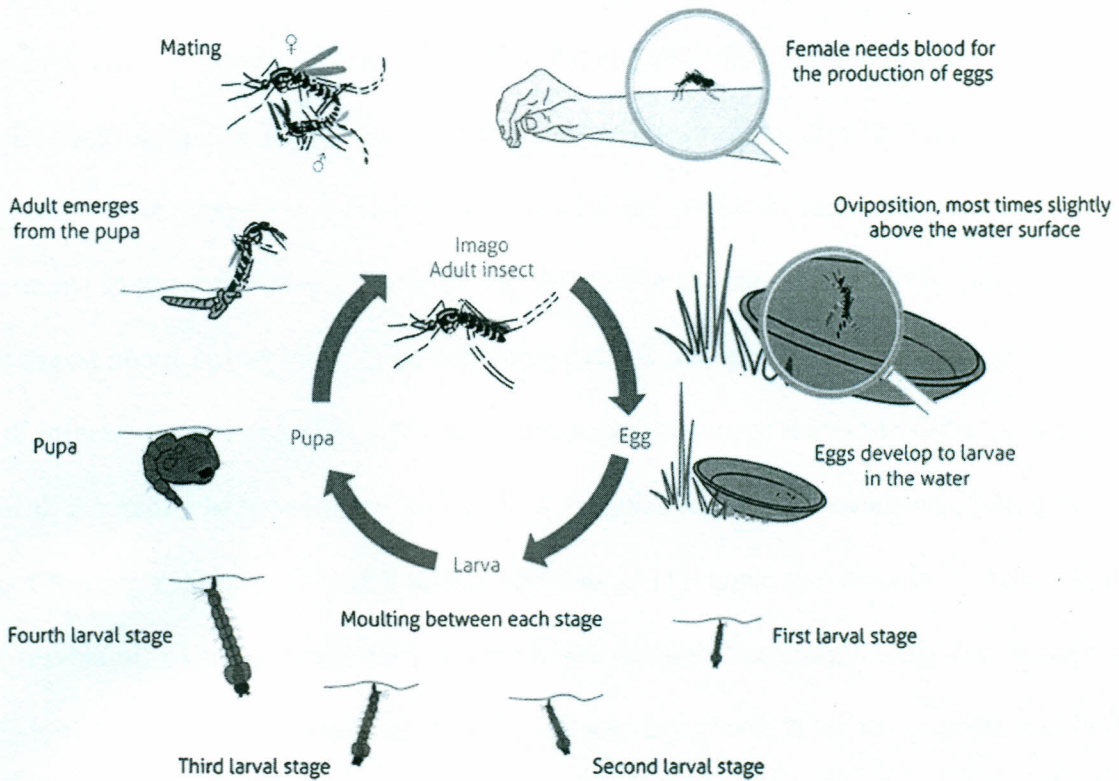


Figure 2.1: Illustration of the life cycle of *Aedes aegypti* mosquito species from the eggs, larvae stage, pupa stage and adult stage.
 (http://www.biogents.com/cms/website.php?id=/en/traps/mosquitoes/tiger_mosquitoes.htm).

2.3 Mechanism of DENV in the Mosquito

The vector of DENV the *Aedes* mosquito species gets infected while taking a blood meal from a viremic person or NHP (WHO, 2013a, 19). The virus attaches to any of the following receptors on the mosquito vector which include glycosphingolipid (GSLs), proteins with chaperone activity, laminin-binding proteins, and other uncharacterized proteins in mosquito cells (Wichit *et. al.*, 2011). For the mosquitoes to be infected, it has to ingest about 1 μ l of blood from a viremic person. The person must have approximately 10^3 infectious units/ml of blood to have one infectious virus transmitted during the blood meal. A viremia in the range of 10^3 to 10^5 is essential for the transmission of dengue virus to a feeding mosquito (Murphy and Whitehead 2011). Once the virus is in the mosquito, it travels all over the body. DENV penetrates through the midgut infection barrier, the midgut escape barrier (intestinal tract) to the salivary glands after an extrinsic incubation period, a process that takes approximately 10 days especially at high ambient temperatures (Guzman *et. al.*, 2010). Once in the midgut the four DENV serotypes attaches to the R67 and R80 (salivary proteins) which are the putative receptors in the mid gut cells of the *Aedes* (*Ae.*) species (Salazar *et. al.*, 2007; Mercado-Curiel *et. al.*, 2006). Thus the mosquito becomes infected ready to transmit the virus into other vertebrate hosts.

Natural transovarial transmission of DENV virus to successive generations under natural conditions has been shown in *Ae. aegypti* in both male and female populations (Thongrunkiat *et. al.*, 2012). This kind of transmission tends to persist without horizontal transmission from vertebrate blood meals (Joshi *et. al.*, 2002). The transovarial transmission of DENV in *Ae. aegypti* mosquitoes, may serve as a natural reservoir for maintenance of DENV in nature (Lee *et. al.*, 1997; Joshi, Mourya, and

Sharma 2002). Transovarial transmission also provides a mechanism to allow DENV to survive dry or cold seasons or the temporary absence of non-immune vertebrate hosts (Thongrungrat *et al.*, 2011). The re-emergence of DENV from inter epidemic to epidemic phase of disease onset in a DENV endemic area may be associated with the transovarial transmission of DENV (Rohani *et al.* 2008). Once infected, the mosquito remains infected for life transmitting the virus to susceptible individuals through probing and feeding (WHO Report, 2013). During blood meal the infected *Aedes* mosquitoes, injects the virus into the bloodstream, which cause virus spillover in the epidermis and dermis (Martina *et al.*, 2009; WHO Report, 2013).

2.4 Mechanism of DENV Pathogenesis in Man

Once in the skin, DENV virus infects cells of the mononuclear phagocyte lineage that include monocytes, macrophages, dendritic cells including the skin resident langerhans cells (Palucka 2000; Wu *et al.*, 2000; Rodenhuis-Zybert *et al.*, 2010). Dengue viruses infect immature dendritic cells through the non-specific receptor dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) (Wu *et al.*, 2000). Other mammalian dengue receptors include sulfated glycosaminoglycans (GAGs), lectins that recognize carbohydrates, GSL, proteins with chaperone activity, laminin-binding proteins, and other uncharacterized proteins (Martina *et al.*, 2009; Wichit *et al.*, 2011). Infected cells mature and migrate from site of infection to lymph nodes, where monocytes and macrophages are recruited, which become targets of infection. Consequently, infection is amplified and virus is disseminated through the lymphatic system. As a result of this primary viremia, several cells of the mononuclear lineage, including blood-derived monocytes, myeloid dendritic cell (DC), splenic and liver macrophages are infected

(Martina *et. al.*, 2009). This migration initiates the cellular and humoral immune responses. Viremia in man lasts approximately 7 to 10 days (Murphy *et. al.*, 2011).

2.5 Replication of DENV

Virions attach to the surface of a host cell and subsequently enter the cell by receptor-mediated endocytosis. Once deep inside the cell, the virus fuses with the endosomal membrane which should be deep inside the cell where the environment is acidic and the endosomal membrane must gain a negative charge. This conditions facilitates the virus envelope to fuse with the endosomal membrane which releases the dengue nucleo-capsid into the cytoplasm of the cell (Li *et. al.*, 2008). Once the genome is released into the cytoplasm, the nucleo-capsid opens to uncoat the viral genome (Kuhn *et. al.*, 2002). The positive-sense RNA is then translated into a single polyprotein that is processed co-translationally and post-translationally by viral and host proteases. The single polypeptide is then cut into ten dengue proteins, and the viral genome is replicated. Genome replication occurs on intracellular membranes (Bartenschlager *et. al.*, 2008). Virus assembly occurs on the surface of the endoplasmic reticulum (ER) when the structural proteins and newly synthesized RNA buds into the lumen of the ER. The newly non-infectious synthesized immature viral and subviral particles are transported through the trans-Golgi network (Mukhopadhyay *et. al.*, 2005). The host protease furin cleaves the immature virion and sub-viral particles. This results into infectious mature particles and sub viral particles that are then released from the cell by exocytosis ready to infect other cells (Mukhopadhyay *et. al.*, 2005).

2.6 Structure and Genomic Organization of dengue virus

Dengue virion is encapsulated within the nucleocapsid and enveloped in a glycoprotein-embedded lipid bilayer. The genomic RNA comprises of a single open reading frame encoding 10 viral proteins: three structural proteins namely, Capsid protein (C), pre-membrane protein (prM) and Envelope protein (E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Fig. 2.2).

The function of C protein is important in virus assembly to ensure specific nucleic acid/viral genome is enclosed in a capsid. The critical role of C is evident from the existence of subviral particles that are released from infected cells but lack C protein and genome RNA (Ferlenghi *et. al.*, 2001). The maturation process of flaviviruses is directed by proteolytic cleavage of the precursor prM, turning inert virus into infectious particles (Ferlenghi *et. al.*, 2001).

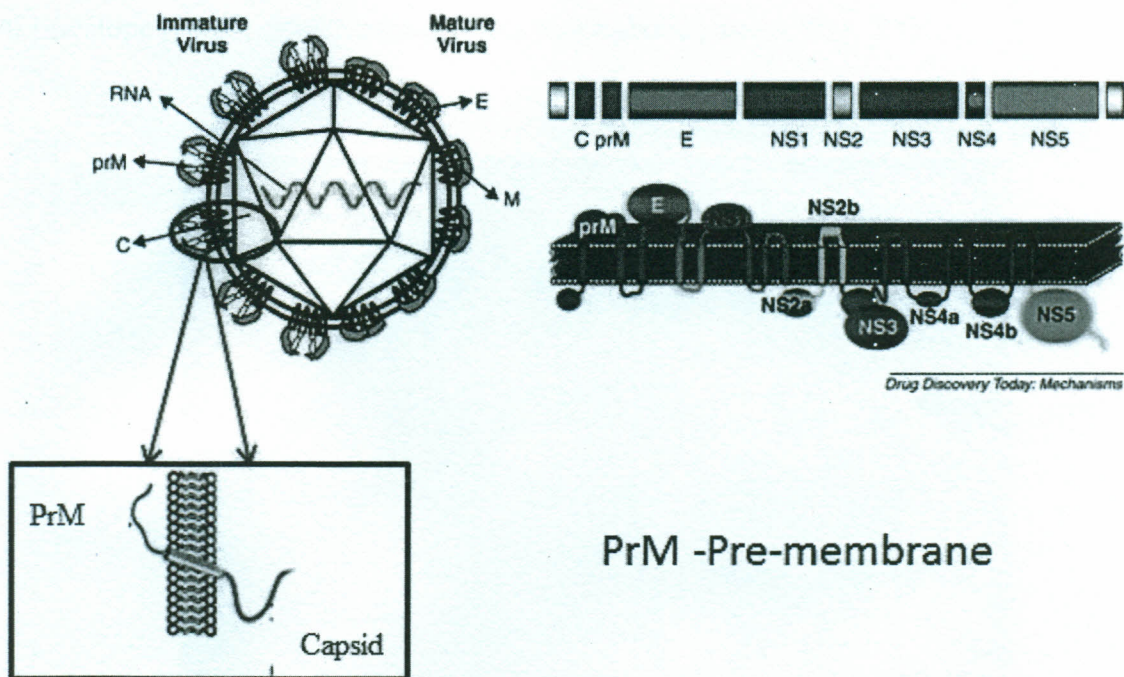


Figure 2.2 : Dengue Virus Structure and Genome Organization.

Diagram Courtesy of Drug Discovery Today, <http://ars.els-cdn.com/content/image/1-s2.0-S1740676511000113-gr1.jpg>

Immature dengue virions have a spiky glycoprotein shell with 60 prM-Envelope arranged in icosahedral protrusions (Fig. 2.3). Matured dengue virions (http://irmosquito.com/irmcd-web/wn_sle4.html) have a smooth glycoprotein shell with 90 Envelope protein dimers arranged in a herringbone pattern (Fig. 2.4).

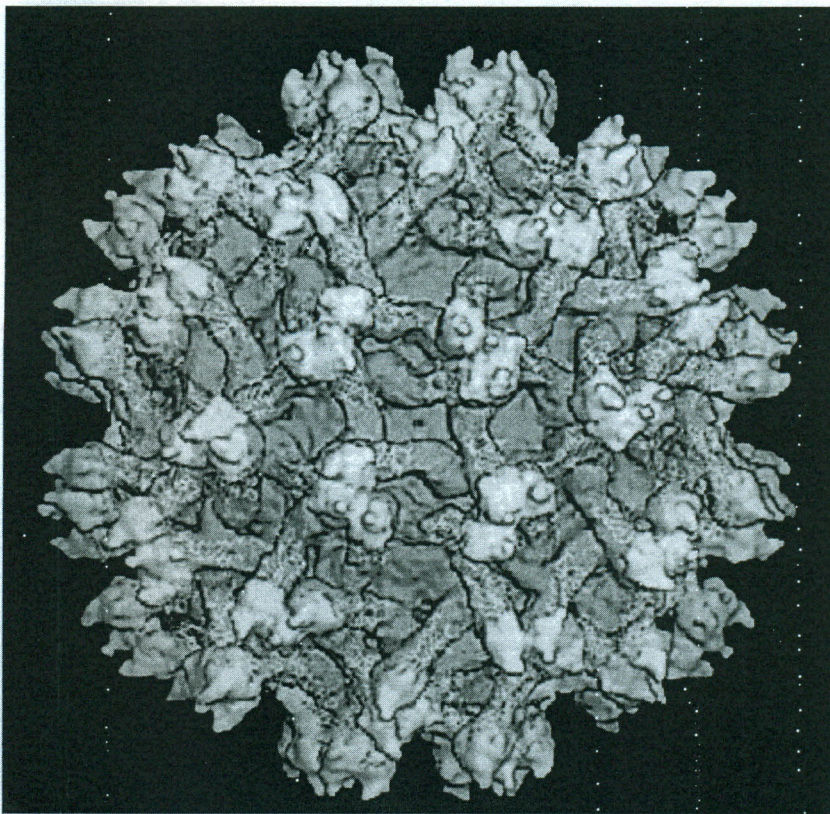


Figure 2.3: Illustration of immature non-infectious dengue viral particle showing the 60 or so trimers, or three prolonged protein spikes, on its surface.

Illustration by Ying Zhang Purdue University, all rights reserved. <http://www.the-scientist.com/?articles.view/articleNo/14953/title/Dengue-Junior/>

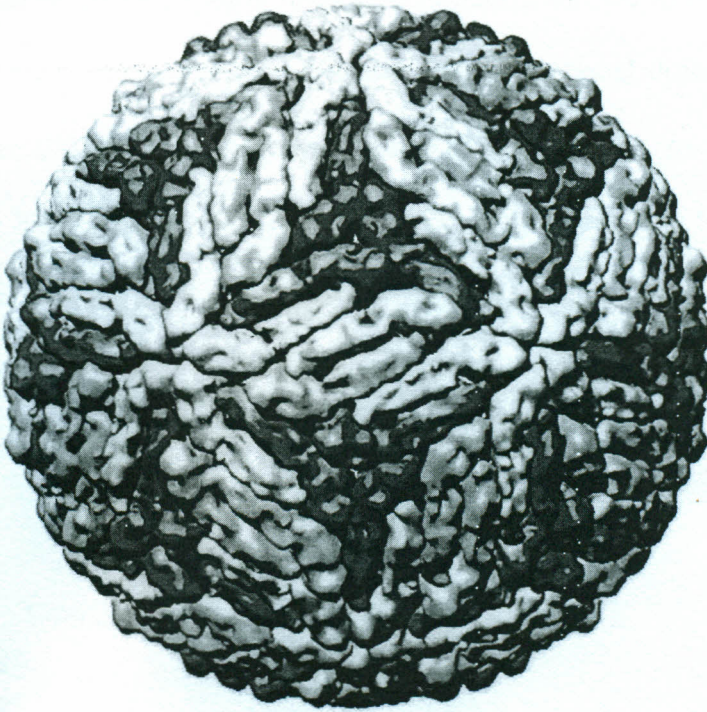


Figure 2.4: Illustration of a mature infectious dengue virus particle showing its smooth surface. Illustration courtesy of Indian River Mosquito Control, http://irmosquito.com/irmcd-web/wn_sle4.html

The E protein is divided into three functional domains, termed as domains I, II, and III. Domain I, the hinge region, is linked to two other functional domains (Fig. 2.5). This region is responsible for the changes in structure of E protein due to variations in external pH. Domain II has a hydrophobic-rich peptide sequence featuring the membrane fusion activity and contributes to E protein dimerization (Modis *et. al.*, 2003; Zhang *et. al.*, 2003). Domain III believed to be involved in the binding to receptor molecules present on the host cell membrane (Fig. 2.5).

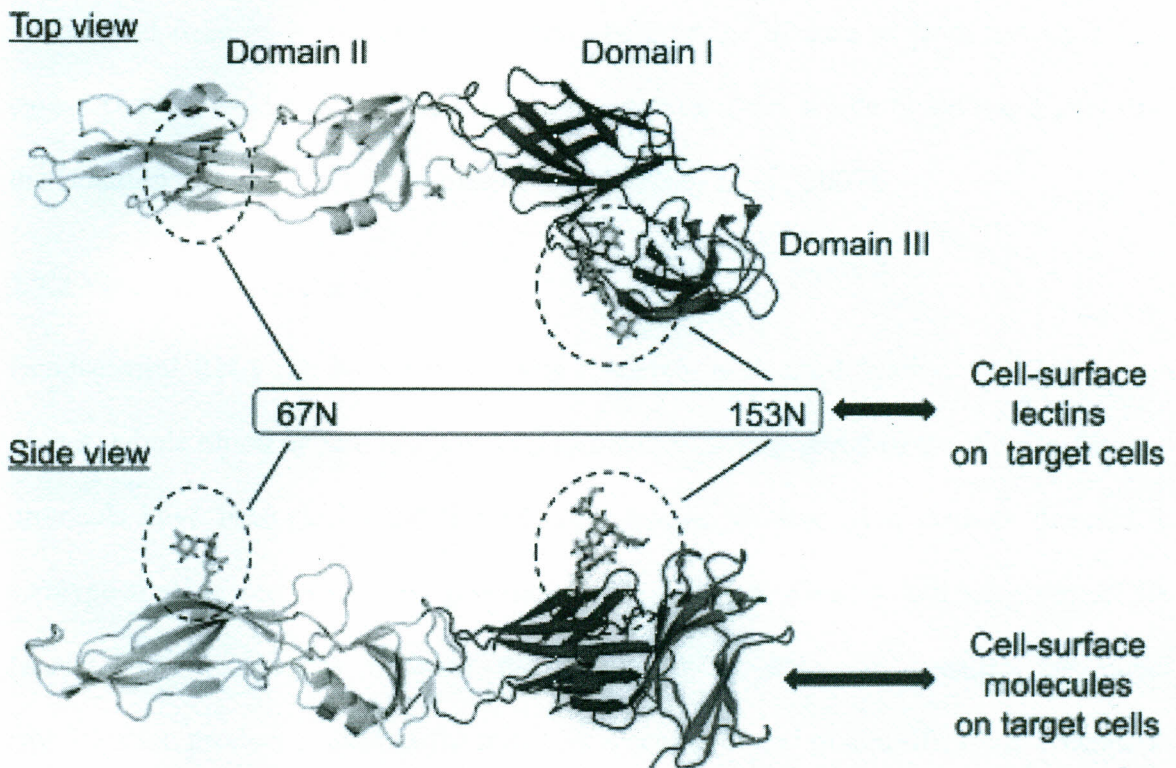


Figure 2.5: Illustration of E protein showing the three domains of DENV E protein. DENV interactions with lectin and other molecules expressed on the host cell surface *N*-Glycan at position 67 is recognized by DC-SIGN, and *N*-glycans at positions 67 and/or 153, may be associated with host lectin proteins such as mannose-binding protein. Until the mid-1920s, dengue was considered a mild disease without a serious public health

impact. This changed dramatically with its increasing prevalence and the appearance of dengue fever, dengue hemorrhagic fever (DHF) and associated shock syndrome, which can be fatal (Monath, 1994).

2.7 Dengue Diagnosis

2.7.1 Virus Detection

Dengue virus can be isolated by the inoculation of diagnostic samples into mosquitoes, cell culture or intra-cerebral inoculation of suckling mice. Whole blood, serum or plasma is collected from patients during the acute phase of the disease or from tissues in fatal cases. There is evidence that the virus isolation rates from whole blood are higher than the isolation rates from serum or plasma (Klungthong *et. al.*, 2007).

2.7.2 Viral RNA Detection

Dengue viral RNA can be detected using a nucleic acid amplification test (NAAT) on tissues, whole blood or sera taken from patients in the acute phase of the disease. Various protocols have been developed that identify dengue viruses using primers directed to serotype-specific regions of the genome (B. W. Johnson, Russell, and Lanciotti 2005). Nested PCR techniques improve the sensitivity of detection because the initial amplification product is used as the target for a second round of amplification. Precaution should be taken when performing nested PCR to prevent false-positive results that can occur as a result of contamination. In situ PCR can be carried out on tissue slides (Peeling *et. al.*, 2010).

2.7.3. Antigen Detection

2.7.3.1. NS1-based Assays.

This is used to diagnose dengue infection in the acute stage. However, antigen detection in the acute stage of secondary infections can be compromised by pre-existing virus-IgG immunocomplexes.

New developments in enzyme-linked immunosorbent assay (ELISA) and rapid immunochromographic assays that target non-structural protein 1 (NS1) have shown that high concentrations of this antigen can be detected in patients with primary and secondary dengue infections up to 9 days after the onset of illness (Hunsperger *et. al.*, 2009). There is a good correlation between NS1 serotype-specific IgG as determined by ELISA and plaque reduction neutralization test (PRNT) results, but the performance and utility of these NS1-based tests require additional evaluation (Peeling *et. al.*, 2010).

2.7.4. Serological Methods

The acquired immune response to dengue virus infection consists of the production of immunoglobulins (IgM and IgG) that are mainly specific for the virus envelope (E) protein. During a primary dengue infection, IgM response is typically higher titre and more specific than during secondary infections. The titre of the IgG response is higher during secondary infection than during primary infection. Serology cannot be used to identify dengue serotypes following a recent infection because the antibodies produced following a primary dengue infection often demonstrate some degree of cross-reactivity with other dengue virus serotypes. Antibodies formed following secondary dengue infections are strongly cross-reactive within the dengue group and also usually crossreact

with other flaviviruses (Innis *et. al.*, 1989).

2.7.4.1 IgG-based assays.

Dengue IgG assays is used for the detection of past dengue infections and current infections if paired sera are collected within the correct time frame to allow the demonstration of sero-conversion between acute and convalescent serum samples. IgG avidity assays can be used to determine whether an infection is a primary or a secondary infection, based on the principle that the avidity of IgG is low after primary antigenic challenge but matures slowly within the weeks and months after infection. The IgG-based ELISA exhibits the same broad cross-reactivity with other flaviviruses.

2.7.4.2. IgM-based assays.

The detection of dengue-specific IgM is a useful diagnostic and surveillance tool. IgM is initially detectable between 3 to 5 days post onset of fever in ~50% of hospitalized patients and has a sensitivity and specificity of ~90% and 98%, respectively, when assays are undertaken five days or more after the onset of fever. Dengue-specific IgM is expressed earlier than dengue-specific IgG (Kuno, Gómez, and Gubler 1991).

2.8 Clinical Manifestation of Dengue

Dengue fever is a severe, flu-like illness that affects infants, young children, adults and NHPs, but seldom causes death (WHO Report, 2013). It has been shown that NHPs do not show disease symptoms in DENV infection while in humans it shows clinical symptoms (Bernardo *et. al.*, 2008). It is accepted that a primary infection with one DENV serotype induces long-term protective immunity to re-infection with the homologous serotype but does not confer immunity to infection with other dengue serotypes (Bernardo *et. al.*, 2008). The risk of severe disease from secondary infection increases if

someone previously exposed to one serotype is exposed to a heterologous strain (Guzman *et. al.*, 2010). Dengue should be considered in the differential diagnosis of all febrile patients living or with a history of travel to the tropics and subtropics in the 2 weeks before symptom onset. The incubation period is typically 4–7 days (range, 3–14 days). The earliest change on clinical/laboratory observations of dengue infection include reduced white cell count (leucopenia), the platelet count does not go low but decreases with time and metabolic acidosis (Low *et. al.*, 2011; Ranjit *et. al.*, 2011).

2.8.1 Dengue Fever Symptoms

Dengue fever should be suspected when a high fever (40°C/ 104°F) is accompanied by two of the following symptoms: severe headache, pain behind the eyes (retro-orbital pain), muscle pains (myalgia) and joint pains (arthralgia), nausea, vomiting, swollen glands or rash and positive tourniquet test is observed. The rash usually appears as the fever subsides and lasts 2–4 days. The rash is either macular or maculopapular and generalized, often confluent with small patches of normal skin, and it may become scaly and pruritic. Other signs and symptoms include flushed skin, usually during the first 24–48 hours (Sirivichayakul *et. al.*, 2012; WHO, 2013). Symptoms of dengue fever will usually last for 2–7 days, after an incubation period of 4 to 10 days after a bite from an infected mosquito (Gregory *et. al.*, 2010; WHO, 2013).

2.8.2 Dengue Hemorrhagic Fever

DHF may have complications due to plasma leaking, fluid accumulation, respiratory distress, severe bleeding, or organ impairment thus making it fatal. In adults, the degree of capillary permeability under normal physiological conditions is less than that for children which may in turn contribute to a reduced risk of DHF in dengue infected

children (Low *et. al.*, 2011). Warning signs of DHF may occur 3–7 days after the first symptoms in conjunction with a decrease in temperature (below 38°C/ 100°F) and include: severe abdominal pain, persistent vomiting, rapid breathing, gingival bleeding, epistaxis, petechiae, fatigue, restlessness, blood in vomit and thrombocytopenia (platelets $<150,000\text{mm}^3$) (Guilarde *et. al.*, 2008; WHO, 2013).

Other symptoms of severe dengue infection have been listed which include diarrhea during day 4–6 of illness and hepatomegaly (Increased size of liver). The next 24–48 hours of the critical stage can be lethal; proper medical care is needed to avoid complications and risk of death (Biswas *et. al.*, 2012; WHO, 2013).

In young children the symptoms of dengue infections are similar to those of adult infection but also have the following; poor capillary refill, cold extremities, hypotension, thrombocytopenia and the absence of cough is associated with dengue infection (Gregory *et. al.*, 2010; Sirivichayakul *et. al.*, 2012; Biswas *et. al.*, 2012).

Neurological appearance of dengue virus infection is a rare occurrence in disease progression. Viral antigens, specific IgM antibodies, and the intrathecal synthesis of dengue antibodies have been well shown in cerebrospinal fluid. This has brought about the neurological manifestations that include encephalitis, encephalopathy, meningitis, Guillain-Barré syndrome, myelitis, acute disseminated encephalomyelitis, polyneuropathy, mononeuropathy, and cerebromeningeal hemorrhage (Solomon *et. al.*, 2000; Pancharoen *et. al.*, 2001; Puccioni-Sohler *et. al.*, 2012; Rao *et. al.*, 2013).

2.9 Management of Dengue Infections

There is no specific medication for dengue infection treatment. Dengue is treated symptomatically. Persons who think they have dengue should use analgesics (pain

relievers) with acetaminophen and avoid those containing ibuprofen, Naproxen, aspirin or aspirin containing drugs. They should also rest, drink plenty of fluids to prevent dehydration, avoid mosquito bites while febrile and consult a physician (WHO Report, 2009).

As with dengue, there is no specific medication for DHF. If a clinical diagnosis is made early, a health care provider can effectively manage DHF using fluid replacement therapy. Adequate management of DHF generally requires hospitalization (WHO Report, 2009).

2.9.1 Dengue Vaccine Strategies

Currently there is no licensed drug or vaccine for dengue infections (WHO, 2013). However there are many ongoing dengue vaccine initiatives that incorporate all the four serotypes. The year 2012 saw an introduction of seven candidate vaccine undergoing clinical trials (Simmons *et. al.*, 2012). Among these dengue vaccine studies the most advanced vaccine is the Sonafi (Lyon, France) tetravalent dengue vaccine candidate (Guy *et. al.*, 2011). This vaccine candidate comprise of four recombinant, live, attenuated vaccines based on a yellow fever vaccine 17D backbone, each expressing the pre-membrane and envelope genes of one of the four dengue virus serotypes (Guy *et. al.*, 2011; Simmons *et. al.*, 2012). This vaccine candidate is in its third phase of clinical trial in endemic areas (Simmons *et. al.*, 2012). The prospect of licensing effective tetravalent vaccines against human DENV raises the potential for the control or even eradication of the human-DENV transmission cycle. However, if additional DENV serotypes that have not yet come forward into human transmission cycles already exist in as-yet-undetected sylvatic cycles, there could be major implications for the long-term control of dengue

infections using the vaccines that are currently under development (Vasilakis *et. al.*, 2011).

2.10 Epidemiology of Dengue Virus

The World Health Organization (WHO) estimates that 50 million to 100 million infections of dengue viruses occur every year all over the world (WHO, 2013). However, recent studies estimate that the dengue infections have risen up to 390 million infections annually (Bhatt *et. al.*, 2013). Dengue fever has been reported in all of Africa, South and Central America (Cuba, Texas), Asia (Middle East, Indonesia) and Australia (Nielsen, 2009). Recently, Bhatt and others estimated that the number of total infections in Africa in 2010 was between 10.5 to 22.5 million infections. The epidemiology of dengue fever in Africa is poorly understood (Bhatt *et. al.*, 2013). However, cases of dengue fever has been reported in several parts of Africa which include; Central Africa, Cameroon in 2006 (Blaylock *et. al.*, 2011) and Gabon in 2007 where it caused simultaneous outbreaks of DENV 2 infections (Caron *et. al.*, 2012). It has also been reported in West African countries Senegal, Mali and Togo. In Southern Africa outbreaks have occurred in Namibia and Zambia (Were, 2012). In Eastern Africa dengue fever has been reported in Kenya (B. K. Johnson *et. al.*, 1982), Ethiopia, Uganda, Tanzania (Were, 2012), Sudan (Abdallah *et. al.*, 2012) and Somalia (Kanesa-thasan *et. al.*, 1998). In 1982 there was a report of outbreak in Kenya at the coastal region where DENV 2 antibodies was detected in human serum samples (Sang and Dunster, 2001; Mease *et. al.* 2011). This outbreak in Kenya was believed to have spread from the Seychelles outbreak that occurred between 1977 and 1979 (Metselaar *et. al.*, 1980).

An outbreak of dengue fever was again reported in 2011 Mandera, in the northern part of Kenya where 5,000 people were affected within a few weeks according to health officials

(Unpublished, Global health policy report, 2011; 2012). However, the most recent dengue outbreak in Kenya was reported in February, 2013 in the Coastal region, Mombasa Island and a few cases in Kwale and Kilifi counties where 15 cases were confirmed (Unpublished).

2.11 Zoonosis

Among the known emerging infections, the proportion of zoonotic infections accounts for about 75%, indicating that the human–animal interface presents a risk for emergence (Taylor *et. al.*, 2001). There has been a distinct transition in human environmental and inter-population interactions that have radically changed the spectrum and causes of infectious diseases in human population. The invasiveness of human activity into all environments and disruption of the ecosystems, globalization and urbanization are reshaping the relations between humans and microbes (McMichael, 2004).

New host species that are not susceptible to a pathogen or micro-organisms can be made susceptible and the pathogen can be efficiently spread (Species jump). Species that initiates a first human infection by a new agent is frequently brought about through contact between a pathogen and human. For species jump to occur, the pathogen has to break various biological barriers e.g interspecies barrier to be able to sustain its existence in human to human transmission. Viruses can attain the ability to jump species through the acquisition of genetic changes and closely related host species that allow infection, replication within new host cells and successful transmission to other hosts or vector organisms (Parrish *et. al.*, 2008, Streicker *et. al.*, 2010).

Zoonosis with wildlife reservoir which includes NHPs presents a great public health problem. NHPs being the closest living relatives of man, have been known to harbor

infectious agents that can be transmitted from animals to man. This is due to the fact that in the recent past, there has been a lot of interactions and close contacts of man with NHPs where both share disease vectors. In addition, transportation of animals and vectors from one region to the other has led to increased chances of zoonoses (Acha and Szyfres, 2003). Some of these agents that have been reported to be zoonotic include viruses from the family flaviviridae (Dengue virus), poxviridae (monkey pox virus), togaviridae (Chikungunya, yellow fever) and among other agents including bacterial and fungal infections. The following viruses have been associated with the following species of monkey: Herpes virus simiae (B virus)- Rhesus monkey, Monkey pox- *Cercopithecus ascanis*, *C. nigrovidis*, *C. petaurica*, Chikungunya- *Papio ursinus*, *Cercopithecus aethiops*, *C. ascanis*, Yellow fever-*Cercopithecus aethiops* (Brack 1987; Wolfe *et. al.*, 1998; Karesh *et. al.*, 2005). Cases of sylvatic dengue virus infections have also been reported in West Africa and Malaysia. The Malaysian strain was isolated from a 27 year old male returning to Spain after visiting his home country Guinea Bissau (Teoh *et. al.*, 2011). Here in Kenya, there have so far been no reported cases associated with dengue infections from the sylvatic strains.

Zoonotic infections have different transmission routes. These routes include; through vectors like mosquitoes (*Plasmodium* spp. and *Aedes* spp.) and ticks, aerosol, oral, direct contact and fomites (Michalak *et. al.*, 1998, Hahn *et. al.*, 2000, Tei *et. al.*, 2003, Hunter and Thompson 2005).

Understanding the epidemiology of how dengue virus serotypes circulate has important public health implications in understanding virus transmission, disease severity, vector control, and development of a safe and efficacious vaccine. There is also need to be

vigilant about the re-emergence of dengue fever and dengue hemorrhagic fever from the sylvatic strains.

CHAPTER THREE: MATERIALS AND METHODS

3.1 Study Site

Study sites were chosen due to the high numbers of non-human primates population living in close proximity to the human population. This increases the risk of zoonotic infections from animals to human populations.

Sampling locations included Nguruman (1°51 S, 36°47 E) and Namanga (2°33 S, 36°47 E) in Kajiado county, Kibwezi (2°25 S, 37°58 E) in Makueni county, Kitale (1°01 N, 35°00 E) in Trans Nzoia county, Kakamega (0°30N S, 34°35 E) in Kakamega county, Nairobi (1°17 S, 36°49 E) in Nairobi county, Aberdares (0°25 S, 36°57E) in Nyeri county and Laikipia (0°5N 36°40 E) in Laikipia county Kenya.

Kakamega and Kitale were chosen because there was the presence of *Aedes* species detected in this areas (Lutomiah *et. al.*, 2013). Makueni county was chosen because of its close proximity to the coastal region where *Aedes* species are also detected (Tabachnick *et. al.*, 1979).

3.2 Study Design

This was a cross sectional study. Blood was collected at one point in time from wild caught NHPs (AGMs and olive baboons).

3.2.1 Sampling Technique

The NHPs were trapped using rectangular traps (1.5m high and 0.9m wide) made from iron bars, wire mesh and weighted trap doors designed by the Institute of Primate research (IPR) staff. The NHPs were baited for a week using maize cobs, carrots and bananas. The monkeys then became habituated. The traps were set in the evening after the animals had scampered to their sleeping site with the aim of capturing them the next

day (Jeneby *et. al.*, 2002; Eley *et. al.*, 2005). This technique gave an equal chance for all the animals regardless of their gender or age to have an equal opportunity to feed and thus trapped using the rectangular animal traps. The trapped animals were transferred to their holding cages and transported to Institute of Primate Research quarantine area.

3.2.2. Sample Size Determination

The sample size was determined using the Fischer *et. al.*, 1998 method.

$$N = \frac{Z^2 P(1-P)}{D^2}$$

Where:

N= Minimum sample size required

Z= 1.96 standard error

P= Postulated prevalence (10%).

D= 0.05 the inverse of 95% confidence limit (the allowable error)

$$N = \frac{1.96^2 \times 0.1(1-0.1)}{0.05^2} = 138.3 \text{ samples}$$

Therefore 287 non-human primate samples was used in this study.

3.3 Blood Samples Collection

Quarantined NHPs were removed from their holding sites after anaesthetization with 1:3 mixture of xylazine (2%) (20 mg/ml; Agrar): Ketamine hydrochloride (10%) and dosage of 0.1ml/Kg body weight (100 mg/ml; Agrar, 3760 AL Soest, Holland) (Suleman *et. al.*, 1999). Bolus intramuscular injection of the mixture was used for both African green monkeys and olive baboons. Individual age was determined by the presence and size of molar teeth in males and in females, by the developmental stage of mammary glands.

Venous blood, 5ml was obtained by femoral veni-puncture in sterile 9ml serum tubes (Greiner bio-one, France) and kept on ice. The blood samples were transported back to the laboratory. Blood was processed immediately after collection for serum. The clotted blood was centrifuged at 3,000 rpm for 30 min, sera aliquoted, and preserved at -20°C until required for sandwich IgG antibody enzyme-linked immunosorbent assay (Ab-ELISA) and at -70°C for RT-PCR (Jeneby *et. al.*, 2002). The animals were then taken back to their holding cages for recovery.

3.4 Detection of Antibodies against DENV 2 by ELISA

3.4.1 Viral Antigen Preparation

The prototype strain DENV-2 New Guinea C, was grown in suckling mouse brain and concentrated by the sucrose-acetone method.

3.4.1.1 Sucrose-Acetone Virus Concentration/Extraction Method

Mice were inoculated by the intra-cerebral route for the production of brain antigens; the dose given was 0.02 ml of a 10^2 dilution of stock infectious suckling-mouse brain. The age of the mice used was 1- to 2-day old mice because the incubation period of dengue virus is long.

The brains were harvested at a time when the mice started showing signs of disease and death. The mice were anesthetized with ether and partially exsanguinated by cutting through the chest wall with a small scissors, after which the brains were removed and transferred into a small, weighed, glass preparation dish (5 x 3 cm) with fitted glass cover held in a bath of CO_2 /dry ice and alcohol. After determination of the brain weight, the container was sealed with Scotch and adhesive tape and stored in a CO_2 ice cabinet. The

infected suckling-mouse brain was homogenized with 4 volumes of chilled 8.5 per cent aqueous solution of sucrose. The whole homogenate was added drop wise, with brisk mechanical stirring, to 20 volumes of chilled acetone. The acetone was in a proportion of 20 volumes to 1 volume of homogenate. The mixture was shaken vigorously and centrifuged for 5 minutes at 1,980g. The milky supernatant fluid was aspirated off; the sediment at this stage was a pink, gummy mass which was tightly adherent to the bottom of the centrifuge bottles. A volume of fresh acetone equal to that originally used was added to each bottle, and the preparations were allowed to stand in ice baths for at least 1 hour to dehydrate the sediment. After sufficient time, the sediment was readily reduced to a fine suspension by use of a thick glass rod. The bottles were then centrifuged at 1,980g for 5 minutes. The supernatant fluid was discarded and the sediments from all bottles were pooled in one with fresh acetone. Centrifugation at 1,980g for 5 minutes was carried out. Extraction was repeated three times, once with a mixture of equal parts of acetone and ether and twice with ether alone. The sediment was dried by evaporating the volatile solvents (ether) via a water pump by clamping the open rubber tubing and placing the flask in a pan of hot water. At the end of the procedure, the dry residue in the flask was decontaminated by autoclaving.

To the dry powder, a volume of saline was added which was equal to 0.4% of the total volume of homogenate used. Owing to the presence of the sucrose, this preparation rehydrated more readily and the antigen preparation was completed after staying overnight in ice. The antigen was then centrifuged for 0.5 to 1 hour at 10,000g. The supernatant fluid was then DENV 2 antigen (Clarke and Casals, 1958).

3.4.2 Sandwich Antibody ELISA for Detection of Anti-dengue IgG antibodies

The exposure levels of the animals to DENV were detected using specific anti dengue immunoglobulin Gamma (IgG). The detection of IgG antibodies is one technique used for laboratory diagnosis of dengue. In humans IgG appears within 5-7 days of the disease, reaching the highest titers during the third week of the disease. IgG titers decrease without disappearing, maintaining an immunological memory as a mark in the serum or serological scar (Ruebens *et. al.*, 2012).

An amplified sandwich ELISA system was used to detect anti-dengue IgG antibodies from the NHPs. Each polystyrene 96 well ELISA plate (NUNC™, Denmark) was coated with 4G2 anti-flavivirus monoclonal antibody at a concentration of 5ug/ml (Sigma, USA). This was then incubated at 37°C for 2 hr. The plates were washed using phosphate buffered saline containing 0.05% Tween 20 (PBS/Tween 20). Blocking was done using 5% skimmed milk in 1X phosphate buffered saline (1X PBS) for 1 hr at 37°C. The plates were washed with PBS/tween 20. The viral antigen was added and then the plates were incubated overnight at 4°C. *Flavivirus* antigens (DEN-2) were prepared and provided by Center for Genetic Engineering and Biotechnology (CIGB) of Havana, Cuba. The following day the unbound antigen was washed away using PBS/Tween. Since the antigens were prepared in mouse brain, uninfected mouse brain antigen was used as negative control. Each test serum sample was tested in duplicate. Animal test sera were added at a 1:100 dilution and incubated for 1 hr at 37°C. The plates were washed with PBS/Tween 20 and incubated for 30 min at 37°C with goat anti-monkey IgG peroxidase conjugate (Amersham, UK). Finally, the plates were developed by adding the substrate solution (Phosphate citrate buffer, hydrogen peroxidase and orthophenylenediamine [OPD]) was then added (Appendix 4.0). The reaction was then stopped using 12.5%

sulphuric acid. Absorbance was read at 450nm in a microplate reader (DYNATECH Laboratories, Germany). Sera that gave an optical density (OD) that was at least two times greater than the normal mouse brain negative control were designated as positive sera.

3.5 Dengue RNA Extraction from Serum

Viral RNA was extracted using the ZYMO RESEARCH ZR -RNA Viral RNA extraction Minikit (Inqaba, South Africa) from serum samples positive by ELISA and stored at -70°C. All the samples were put on ice in all the subsequent steps. Briefly, 40 µl of RNA lysis buffer was added to 10 µl samples and mixed well. Samples were centrifuged at 12,000 g for 60 seconds. The lysate was transferred into a zymo-spin™ 111C column in a collection tube. This was centrifuged at 8,000 g for 30 seconds and the flow through was collected in a collection tube. Into the collection tube, 0.8 ml volume of ethanol (95-100%) was added to the flow through and vortexed well. The flow through mixture was transferred to a zymo-spin™ 11C column. This was centrifuged at 12000 g for 60 seconds. The flow through was then discarded and 400 µl of RNA Prep buffer added to the column and centrifuged again at 12000 g for 60 seconds. The flow through was discarded and replaced with a Zymo-spin 11C column back into the collection tube. Into the column 800µl of RNA wash buffer was added followed by centrifugation at 12,000g for 30 seconds. The flow through was discarded and the Zymo-spin™ 11C column was placed into a new collection tube. The wash was repeated as above using 400µl of RNA wash buffer and a spin of 12000 g for 30 secs. The Zymo-spin™ 11C column was span again at 12000g for 129 seconds in the emptied collection tube to ensure complete removal of the RNA wash buffer. The Zymo-spin™ 11C column was placed into an RNase free tube and 25µl of DNase/RNase free water was directly put in the column

matrix and let to stand at room temperature for 60 seconds. Later it was centrifuged at 10000 g for 30 seconds to elute the RNA from the column.

3.6 Reverse Transcription of Extracted Dengue Virus

The extracted viral RNA was immediately transcribed to cDNA as follows. Briefly, RevertAid Premium First Strand cDNA Synthesis kits (Fermentors, European Union) was used for reverse transcription. The kits were brought to room temperature to thaw. All the kit reagents were thoroughly mixed and stored on ice. In a sterile, RNase free tube on ice the following reagents were added: 8µl of nuclease free water, 1 µl of 10mM dNTP mix (to a final concentration of 0.5mM) and 1 µl of random hexamer primer (100 pMol). The above were mixed gently and centrifuged briefly. This was incubated at 65°C for 5 min. Later it was placed on ice until use. To the above reaction, 4 µl of 5X RT buffer (See Appendix 5.0) mixed with 1 µl RevertAid™ Premium Enzyme Mix was added. This was then incubated at the following temperatures 25°C for 10 min and 50°C for 30 min. The reaction was terminated by finally heating at 85°C for 5 min. The reaction product of this first strand cDNA synthesis was used directly for first amplification.

3.6.1 Primary Amplification of the First strand cDNA

The primer used (Appendix 6.3) (D1) 5' TCAATATGCTGAAACGCGCGAGAAACCG-3' and downstream consensus primer (D2) 5'TTGCACCAACAG TCAATGTCTT CAG TTC-3' (Lanciotti *et. al.*, 1992) corresponds to a portion of the capsid and pre-membrane of the four DENV serotypes at base pair 511. The master mix was prepared by addition of 22µl of double distilled nuclease free water, 25 µl of Dream Taq master Manufacturer name (2X), 1 µl of forward primer, 1 µl of reverse primer and 1 µl of the template cDNA. The amplification of copy DNA was done using the DNA Thermal Cycler (Perkin-Elmer® Cetus, Norwilk, USA). The thermocycler was programmed to

incubate at 42°C for 1 hr proceeded by 35 cycles of denaturation at 94°C for 30 seconds. For primer annealing at 55°C for 60 seconds and primer extension at 72°C for 2 min (Lanciotti *et. al.*, 1992).

Agarose gel electrophoresis was used to determine and visualize the presence of *PCR* products. Specific-DNA amplification was confirmed by electrophoresis of 10 µL of first PCR product using 1% agarose gels stained with ethidium bromide and visualized using a transilluminator, BioDo-It.Imaging System (Prado *et. al.*, 2005).

3.6.2 Secondary Amplification of the Second Strand cDNA

To amplify a 119bp of the capsid region of DENV 2, a second round of amplification reaction was performed using amplicons from the first amplification reaction with upstream DENV 2 (D1) 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' and downstream specific primer TS2 5'-CGCCACAAGGGCCATGAACAG-3'.

The master mix (Appendix 6.2) reaction mixture contained all the components described for the initial amplification reaction (Section 3.6.1) with the following exceptions: primer D2 which was replaced with the dengue virus type-specific primers TS2. The PCR was done in the following conditions: 20 cycles of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 1 minute, and primer extension at 72°C for 2 minutes. The amplified product was electrophoresed on 1% agarose gel for the first amplification and 2% agarose gel Tris acetate EDTA buffer (TAE buffer) (Lanciotti *et. al.*, 1992; Prado *et. al.*, 2005). In the reactions positive and negative controls were included in all (Prado *et. al.*, 2005).

3.7 Gel Electrophoresis

Agarose gel electrophoresis was used to determine and visualize the presence of *PCR*

products. Specific-DNA secondary amplification was confirmed by electrophoresis using 2% agarose gels using 10 µL of the amplicons visualized using a transilluminator, BioDo-It.Imaging System (Prado *et. al.*, 2005).

3.8 Ethical Considerations

This study was ethically approved by Institute of Primate Research (IPR) (Kenya) scientific review committee and a permit to trapping the NHPs and collect blood samples from the animals granted by Kenya Wildlife Service, Nairobi. Animal acquisition, care and maintenance were carried out according to the guidelines of Institutional Scientific Review Committee (ISERC) of the Institute of Primate Research (IPR) (Kenya).

3.9 Data Analysis

Dengue exposure rates differences between NHPs species (*P. anubis* and *C. aethiops*), sampling locations (High lands and low lands), age groups (Adults and juvenile) and sexes (male and female) was compared using Chi square test (χ^2) or Fischer's exact test (two sided) at a confidence interval of 95%. GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA) was used for data analysis.

CHAPTER FOUR: RESULTS

4.1 Antibody ELISA

Out of 287 NHPs sampled, 172 were from *C. aethiops* and 115 *P. anubis*. The animals were sampled from the following regions Aberdares, Kakamega, Kibwezi, Kitale, Laikipia, Nairobi, Namanga and Kajiado counties (Table 4.1). Of those sampled, the prevalence of dengue infection in NHPs was at 37.98% (109 out of 287).

Table 4.1: Sero-prevalence of anti-dengue antibodies from selected regions in Kenya detected by ELISA

Location	No. of	Positive for Antibody	Percentage (%) Positive for
	Animals Tested	ELISA	Antibody ELISA
Aberdares	32	17	53.1%
Kajiado	25	8	32%
Kakamega	20	8	40%
Kibwezi	23	10	43.5
Kitale	19	9	47.4
Laikipia	115	41	35.7
Nairobi	28	8	28.6
Namanga	25	8	32
Total	287	109	37.98%

4.1.1 Analysis by Location

The highest prevalence of dengue was detected in Aberdares with a 53.1% (17 out of 32) with the least exposure seen in Nairobi with a 28.6% (8 out of 28) cases respectively. These regions were further divided into low-land and high-land regions. The low-land regions comprised of Kajiado, Namanga and Kibwezi. The high-land regions comprised of Laikipia, Kitale, Kakamega, Nairobi and aberdares. When Chi-square was used to compare rate of infection in the low-land and high-land there was no statistical significant, P value 0.3065.

4.1.2 Analysis by Species

Among the two species sampled in this study, the prevalence in *P. anubis* was 35.7% (41/115) and 39.5% (68/172) for *C. aethiops*. When Chi-square\Fisher's test was applied to compare the infections between the species, there was no statistical significance between the rates of infection between two species, P value = 0.537 (Table 4.2).

4.1.3 Analysis by Age

In relation to the two age groups sampled in the study (Adults and Juveniles), there was no significant difference (P value 0.1132, $\alpha < 0.05$), in the rates of infection despite juveniles at 42.1% (67/159) being more infected (Table 4.2).

4.1.4 Analysis by Gender

In this study more males 38.1% (61/160) compared to females 37.8% (48 /127) sampled were found to have anti-dengue IgG antibodies (Table 4.2). There was no significance difference across gender (P value 1.00 , $\alpha < 0.05$).

Table 4.2: Sero prevalence of dengue antibodies comparing regions, age, gender and species exposure rates.

Variables	Groups	Number Examined (n)	Positive by IgG ELISA	Positive (%)	P value
Regions	Low-lands	73	26	35.6	0.677
	High-lands	214	83	38.8	
Species	<i>P. anubis</i>	115	41	35.7	0.537
	<i>C. aethiopes</i>	172	68	39.5	
Age	Adult	159	67	42.1	0.113
	Juvenile	128	42	32.8	
Gender	Female	127	48	37.8	1.00
	Male	160	61	38.1	

4.2 Polymerase Chain Reaction Results

The presence of dengue virus circulating in non-human primates was confirmed by reverse transcription polymerase chain reaction, PCR (Figure 4.3). This confirmation was done using positive samples from the ELISA. The prevalence of DENV 2 by PCR for *P. anubis* is 4.88% (2/41) and 5.88% (4/68) for *C. aethiops*. DENV 2 was detected by PCR (Table 4.3). There was no statistical significance (P value 1.000).

Agarose gel analysis of the cDNA product from first round of amplification showing characteristics band of general dengue capsid and pre-membrane regions at 511bp (Fig. 4.1). Fig. 4.2 Shows characteristic bands of DENV 2 at 119 bp.

Table 4.3: Results by Reverse Transcription polymerase chain reaction comparing the results from *C. aethiops* and *P. anubis* species.

Species	Location	Positive by RT-PCR	Positive Percentage (%)	Negative Percentage (%)	P value
<i>P. anubis</i>	Laikipia	2	4.88	95.12	1.000
<i>C. aethiopes</i>	Kakamega	1	6.25	93.75	
	Kibwezi	2	20	80.00	
	Kitale	1	11.11	88.89	

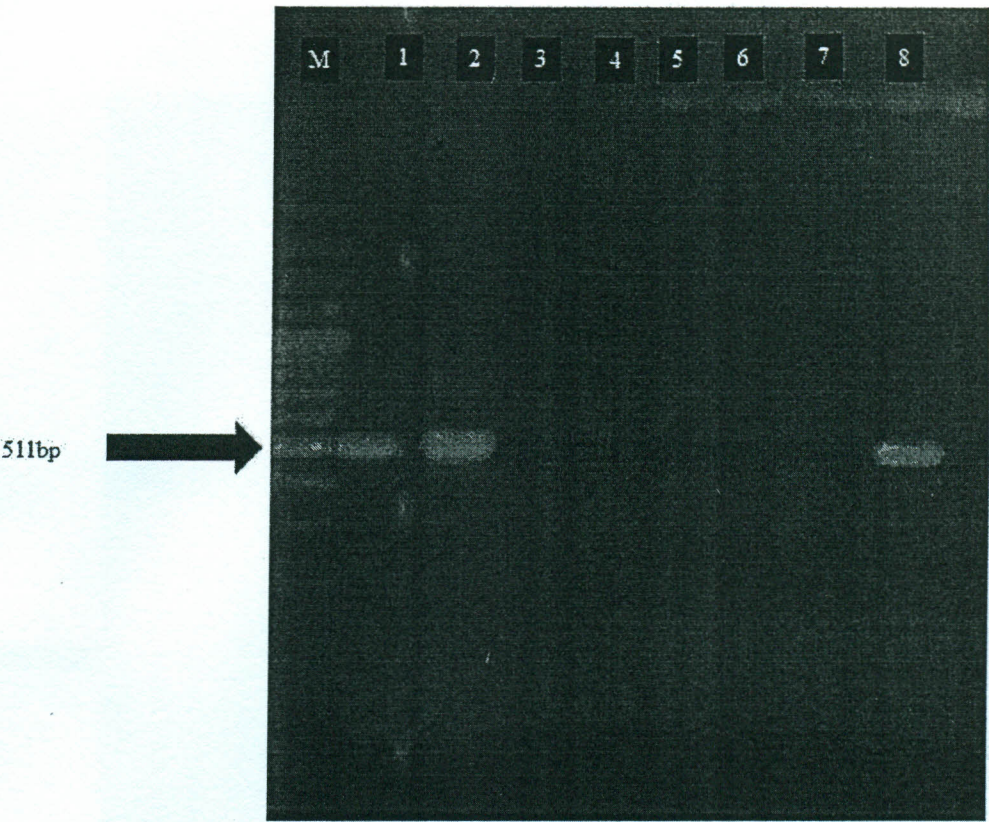


Figure 4.1: Agarose gel analysis of the cDNA product from primary amplification with consensus primers D1 and D2. Positive amplification at approximately 511 bp are shown in lane 1 and 2 (test samples) and lane 8 for positive control. Column 3-7 (Negative test samples). Column M is 100bp molecular Ladder.

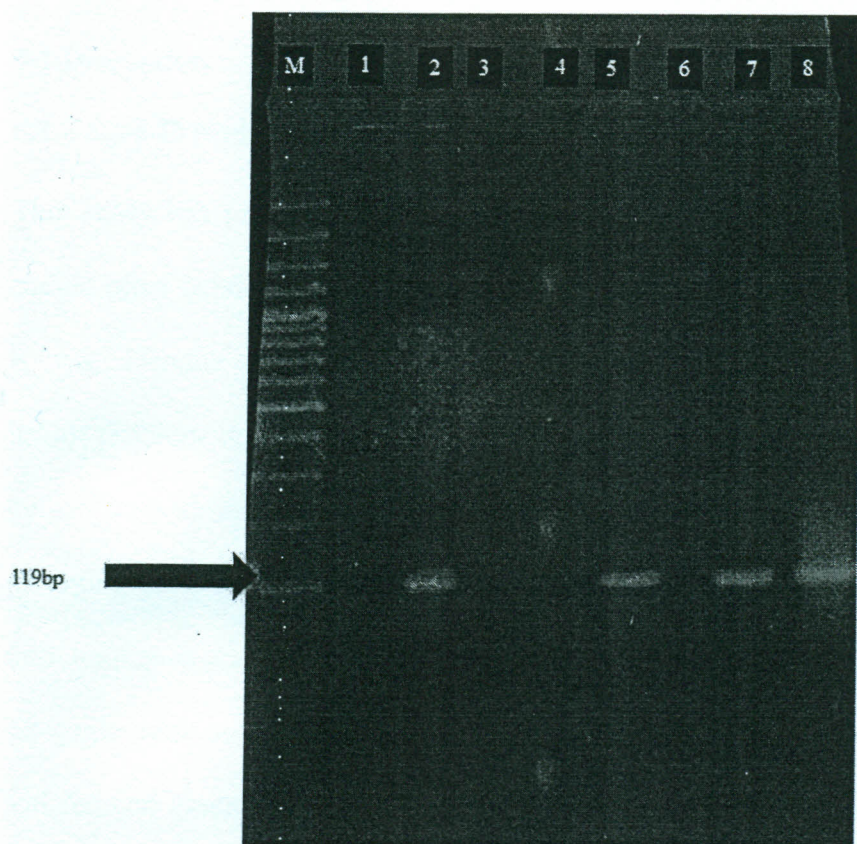


Figure 4.2: Agarose gel analysis of the cDNA product from second secondary amplification with consensus primers D1 and D2. Positive amplification at approximately 119 bp are shown in lane 2, 5 and 7 (test samples) and lane 8 for positive control. Column 1, 3, 4 and 6 (Negative samples). Column M is 100bp molecular Ladder.

CHAPTER FIVE: DISCUSSION, CONCLUSION AND RECOMENDATION

5.1 Discussion

5.1.2 Sero-Prevalence

This study has provided strong evidence of natural exposure to dengue virus in wild caught olive baboons and African Green Monkeys from selected geographical regions in Kenya. Dengue virus infection in the recent years has emerged as an important public health problem in the world. DENV is believed to have evolved as a virus in the non-human primate which is transmitted by the canopy-dwelling arboreal mosquitoes before diverging into the 4 DENV serotypes (DENV-1 to DENV-4), each of which later emerged into human populations (Wang *et. al.*, 2000). Nevertheless, this study has provided serologic evidence of sylvatic dengue virus circulating among wild caught olive baboons and African green monkeys in Kenya. These findings are in consistent with Nakgoi *et. al.*, 2013 who found out that monkey of different species can be naturally infected by DENV (Nakgoi *et. al.*, 2014).

Due to evolutionary change and natural selection patterns of DENV, both sylvatic and endemic strains try to suggest the possibility of future DENV re-emergence from the sylvatic cycle (Vasilakis *et. al.*, 2007). In fact, there have been documented transmission reports of sylvatic dengue virus infecting human populations in West Africa and Malasysia which suggests that sylvatic DENV comes into contact regularly with humans (Franco *et. al.*, 2011). This may be brought about by the movement of arboviruses from sylvatic cycle to human-mosquito-human or an epidemic cycle (Gubler, 1998). This is facilitated by the opportunistic feeding patterns of the competent *Aedes* mosquitoes on man, being a reservoir and amplification host (Vasilakis *et. al.*, 2009). Some other factors

that may lead to the sylvatic DENV spillover include deforestation which has been associated with increased prevalence of several arthropod-borne diseases such as malaria (Volney *et. al.*, 2002; Olson *et. al.*, 2010). Urbanization may also lead to increased chances of exposure to sylvatic DENV. This is because the more man moves into forested areas, the more the chances of being bitten by the *Aedes spp.* which could initiate human-mosquito-human transmission. This type of transmission has been well demonstrated by chikungunya virus outbreak in Europe (Italy) and Africa (Gabon) where human mosquito identical to that of DENV with the same vector was involved (Pagès *et. al.*, 2009). An increase in human population also supports continuous viral transmission (Zanotto *et. al.*, 1996).

The difference in distribution of dengue outbreaks has been associated with climatic change. In addition, human factors like encroachment in forested areas are much more significant (Reiter, 2001). The presence of anti-dengue IgG in the different geographical regions may be attributed to the different weather conditions in the areas which may contribute to either an increase or decrease in the number of mosquitoes (Tipayamongkholgul *et. al.*, 2009). In as much as DENV is related to warm conditions, the virus may be maintained through transovarial transmission during cold seasons. The *Aedes spp.* have been reported in different parts of Kenya which include Laikipia, Kitale and Kakamega which may be a source of sylvatic dengue infections (Evans *et. al.*, 2008; Lutomiah *et. al.*, 2013). There is also a high sero-prevalence in the highland in this study. This may be due to the migration nature of NHPs moving from one locality to the other in search of food. The presence of *Ae. aegypti* and *Ae. africanus* could easily result in sylvatic dengue spilling over to urban areas where *Ae. aegypti* mosquitoes are likely to be

found.

The rate of infection in the different regions, High land 38.78% (83/214) and low lands 35.62% (26/73) region was almost the same with the high lands having a slightly higher exposure rates. This phenomenon may be attributed by climate change because *Aedes* mosquitoes are known to exist in warmer regions (Colón-González *et. al.*, 2013). This phenomenal can further be explained with an example of malaria where in 1970's-1990's, due to climatic changes where highlands areas have become warmer, this has exacerbation malaria cases in this region. Plasmodium sp. are known to exist in warmer areas (Alonso, Bouma, and Pascual 2010). Migration of the NHPs from one area to another in search for food and conducive living conditions may have also contributed to the higher exposure rates in the high lands.

This study has shown that, the prevalence of anti-dengue antibodies in NHPs tends to increase with age, where more adults 42.1% (67/159) than juveniles 32.8% (67/159) were exposed to dengue which is coherent with other studies done in Sri-lanka (de Silva *et. al.*, 1999). These can be explained by the fact that there is gradual exposure in older animals in their life time compared to the juveniles. This trend has also been recorded in human studies in DENV endemic areas in Central India (Ukey *et. al.*, 2010). This illustrates that there might be a more stable rate of ongoing DENV transmission between the arboreal mosquitoes and non-human primates.

In this study, more *C. aethiops* 39.5% (68/170) were exposed to DENV compared to *P. anubis* 35.7% (41/115). This accrues from the habitational nature of *C. aethiops* which are mainly arboreal or semi terrestrial while baboons are terrestrial or ground dwelling. This means that AGMs are more exposed to the *Aedes spp.* mosquitoes which are also

aboreal or mostly found on tree canopies (Anapol *et. al.*, 1996; Patel and Wunderlich 2010).

There was a variation in the rate of exposure in gender, where more males were positive which is consistent with human studies (Mahmood *et. al.*, 2013). These variations can be explained on the basis of difference in the selected population for this study. Furthermore, there is a paradoxical relationship of dengue vector habitat and its biting nature. There may be differences in biting nature of *Aedes* mosquitoes in this part of the world. Females may be more protected owing to some hormones which repel the vector (Guerra-Silveira F. and Abad-Franch F., 2013). These questions need to be explored more.

5.1.2 Molecular Characterization of Dengue Virus

Previous studies have shown that flaviviruses antigens do cross-react between themselves. Due to this variation, this study characterized viral strains by use of RT-PCR (Martin *et. al.*, 2000; Johnson *et. al.*, 2000; Martin *et. al.*, 2002). Findings from this study confirmed circulating dengue 2 virus among Kenyan non-human primates.

5.1.3 Limitations of the Study

This study was limited by convenience sampling of NHPs, it demonstrates a universal exposure of these animals to dengue virus. These findings tend to suggest that sylvatic DENV 2 have a potential for re-emergence in the near future.

5.2 Conclusion

Non-human primates have been associated with zoonosis, as either being potential reservoir host or amplification hosts. Dengue being one of the zoonotic diseases, this study has successfully detected DENV-2 serotype circulating within the non-human primates in Kenya. Sylvatic dengue being an important arboviral infection and a major

cause of morbidity in tropical and subtropical regions, has the potential of causing spill over into the human population through transmission by appropriate *Aedes* mosquito sp.

These findings suggest that:

- I) Sylvatic dengue viruses circulate in non-human primates found in Kenya.
- II) The main serotype circulating in Kenyan non-human primates is Dengue 2 virus.

5.3 Recommendations

1. Strengthening the country surveillance of dengue viruses which will likely become a serious public-health problem in the near future in human population that live in close proximity to animals. This should include reducing human activities in forested areas that is meant for wild life and control of aedes mosquito species which are dengue vectors.
2. Further studies should be carried out to understand the diversity of the other DENV serotypes which may be circulating in Kenyan non-human primates.
3. These strains will be of importance in vaccine developments and advance diagnostic tools.

REFERENCES

- Abdallah, Tajeldin M, Abdel Aziem A Ali, Mubarak S Karsany, and Ishag Adam.** 2012. "Epidemiology of Dengue Infections in Kassala, Eastern Sudan." *Journal of Medical Virology* 84 (3) (March): 500–503.
- Acha, Pedro N., and Boris Szyfres.** 2003. "Zoonoses and Communicable Diseases Common to Man and Animals: Chlamydioses, Rickettsioses, and Viroses". *Pan American Health Org.*
- Alonso, David, Menno J. Bouma, and Mercedes Pascual.** 2010. "Epidemic Malaria and Warmer Temperatures in Recent Decades in an East African Highland." *Proceedings of the Royal Society B: Biological Sciences*, November, rspb20102020. doi:10.1098/rspb.2010.2020.
- Amarasinghe, Ananda.** 2011. "Dengue Virus Infection in Africa." *Emerging Infectious Diseases*, August. doi:10.3201/eid1708.101515.
- Anapol, F, and K Barry.** 1996. "Fiber Architecture of the Extensors of the Hindlimb in Semiterrestrial and Arboreal Guenons." *American Journal of Physical Anthropology* 99 (3): 429–47. doi:10.1002/(SICI)1096-8644(199603)99:3<429::AID-AJPA5>3.0.CO;2-R.
- Bartenschlager, Ralf, and Sven Miller.** 2008. "Molecular Aspects of Dengue Virus Replication." *Future Microbiology* 3 (2): 155–65. doi:10.2217/17460913.3.2.155.
- Bernardo, Lidice, Alienys Izquierdo, Irina Prado, Delfina Rosario, Mayling Alvarez, Emidalys Santana, Jorge Castro, et al.** 2008. "Primary and Secondary Infections of Macaca Fascicularis Monkeys with Asian and American Genotypes of Dengue Virus 2." *Clinical and Vaccine Immunology: CVI* 15 (3): 439–46. doi:10.1128/CVI.00208-07.
- Bhatt, Samir, Peter W. Gething, Oliver J. Brady, Jane P. Messina, Andrew W. Farlow, Catherine L. Moyes, John M. Drake, et al.** 2013. "The Global Distribution and Burden of Dengue." *Nature* 496 (7446): 504–7. doi:10.1038/nature12060.
- Biswas, Hope H., Oscar Ortega, Aubree Gordon, Katherine Standish, Angel Balmaseda, Guillermina Kuan, and Eva Harris.** 2012. "Early Clinical Features of Dengue Virus Infection in Nicaraguan Children: A Longitudinal Analysis." *PLoS Negl Trop Dis* 6 (3): e1562. doi:10.1371/journal.pntd.0001562.
- Brack, Manfred.** 1987. *Agents Transmissible from Simians to Man*. Springer-Verlag.
- Cardosa, Jane, Mong How Ooi, Phaik Hooi Tio, David Perera, Edward C. Holmes, Khatijar Bibi, and Zahara Abdul Manap.** 2009. "Dengue Virus Serotype 2 from a Sylvatic Lineage Isolated from a Patient with Dengue Hemorrhagic Fever." *PLoS Negl Trop Dis* 3 (4): e423. doi:10.1371/journal.pntd.0000423.

Carey, D E, O R Causey, S Reddy, and A R Cooke. 1971. "Dengue Viruses from Febrile Patients in Nigeria, 1964-68." *Lancet* 1 (7690): 105-6.

Clarke, D H, and J Casals. 1958. "Techniques for Hemagglutination and Hemagglutination-Inhibition with Arthropod-Borne Viruses." *The American Journal of Tropical Medicine and Hygiene* 7 (5): 561-73.

Da Silva-Nunes, Mônica, Vanda A F de Souza, Cláudio S Pannuti, Márcia A Sperança, Ana Carolina B Terzian, Maurício L Nogueira, Anna M Y Yamamura, et al. 2008. "Risk Factors for Dengue Virus Infection in Rural Amazonia: Population-Based Cross-Sectional Surveys." *The American Journal of Tropical Medicine and Hygiene* 79 (4): 485-94.

De la C Sierra, B, G Kourí, and M G Guzmán. 2007. "Race: A Risk Factor for Dengue Hemorrhagic Fever." *Archives of Virology* 152 (3): 533-42. doi:10.1007/s00705-006-0869-x.

Domingo, Cristina, Matthias Niedrig, Joaquim Gascón, Gustavo Palacios, Noelia Reyes, María José Malo, Ole Wichmann, et al. 2011. "Molecular Surveillance of Circulating Dengue Genotypes Through European Travelers." *Journal of Travel Medicine* 18 (3): 183-90. doi:10.1111/j.1708-8305.2011.00501.x.

Eley, R. M., S. C. Strum, G. Muchemi, and G. D. F. Reid. 2005. "Nutrition, Body Condition, Activity Patterns, and Parasitism of Free-ranging Troops of Olive Baboons (*Papio Anubis*) in Kenya." *American Journal of Primatology* 18 (3): 209-19. doi:10.1002/ajp.1350180304.

Evans, A., F. Gakuya, J. T. Paweska, M. Rostal, L. Akoolo, P. J. Van Vuren, T. Manyibe, et al. 2008. "Prevalence of Antibodies against Rift Valley Fever Virus in Kenyan Wildlife." *Epidemiology and Infection* 136 (9): 1261-69. doi:10.1017/S0950268807009806.

Ferlenghi, I., M. Clarke, T. Ruttan, S. L. Allison, J. Schlich, F. X. Heinz, S. C. Harrison, F. A. Rey, and S. D. Fuller. 2001. "Molecular Organization of a Recombinant Subviral Particle from Tick-Borne Encephalitis Virus." *Molecular Cell* 7 (3): 593-602.

Franco, Leticia, Gustavo Palacios, José Antonio Martinez, Ana Vázquez, Nazir Savji, Fernando De Ory, María Paz Sanchez-Seco, Dolores Martín, W Ian Lipkin, and Antonio Tenorio. 2011a. "First Report of Sylvatic DENV-2-Associated Dengue Hemorrhagic Fever in West Africa." *PLoS Neglected Tropical Diseases* 5 (8): e1251. doi:10.1371/journal.pntd.0001251.

Franco, Leticia, Gustavo Palacios, José Antonio Martinez, Ana Vázquez, Nazir Savji, Fernando De Ory, María Paz Sanchez-Seco, Dolores Martín, W. Ian Lipkin, and Antonio Tenorio. 2011b. "First Report of Sylvatic DENV-2-Associated Dengue

Hemorrhagic Fever in West Africa." *PLoS Negl Trop Dis* 5 (8): e1251.
doi:10.1371/journal.pntd.0001251.

Gregory, Christopher J., Luis Manuel Santiago, D. Fermin Arguello, Elizabeth Hunsperger, and Kay M. Tomashek. 2010. "Clinical and Laboratory Features That Differentiate Dengue from Other Febrile Illnesses in an Endemic Area--Puerto Rico, 2007-2008." *The American Journal of Tropical Medicine and Hygiene* 82 (5): 922-29.
doi:10.4269/ajtmh.2010.09-0552.

Gubler, D J. 1998. "Dengue and Dengue Hemorrhagic Fever." *Clinical Microbiology Reviews* 11 (3): 480-96.

Gubler, Duane J. 2002. "Epidemic Dengue/dengue Hemorrhagic Fever as a Public Health, Social and Economic Problem in the 21st Century." *Trends in Microbiology* 10 (2): 100-103.

Guilarde, Adriana O., Marilia D. Turchi, Joao Bosco Siqueira Jr, Valeria C. R. Feres, Benigno Rocha, Jose E. Levi, Vanda A. U. F. Souza, Lucy Santos Vilas Boas, Claudio S. Pannuti, and Celina M. T. Martelli. 2008. "Dengue and Dengue Hemorrhagic Fever among Adults: Clinical Outcomes Related to Viremia, Serotypes, and Antibody Response." *Journal of Infectious Diseases* 197 (6): 817-24.
doi:10.1086/528805.

Guy, Bruno, Beatrice Barrere, Claire Malinowski, Melanie Saville, Remy Teyssou, and Jean Lang. 2011. "From Research to Phase III: Preclinical, Industrial and Clinical Development of the Sanofi Pasteur Tetravalent Dengue Vaccine." *Vaccine* 29 (42): 7229-41. doi:10.1016/j.vaccine.2011.06.094.

Guzman, Maria G., Scott B. Halstead, Harvey Artsob, Philippe Buchy, Jeremy Farrar, Duane J. Gubler, Elizabeth Hunsperger, et al. 2010. "Dengue: A Continuing Global Threat." *Nature Reviews Microbiology* 8 (December): S7-16.
doi:10.1038/nrmicro2460.

Hahn, Beatrice H., George M. Shaw, Kevin M. De, Cock, and Paul M. Sharp. 2000. "AIDS as a Zoonosis: Scientific and Public Health Implications." *Science* 287 (5453): 607-14. doi:10.1126/science.287.5453.607.

Hopp, Marianne J., and Jonathan A. Foley. 2001. "Global-Scale Relationships between Climate and the Dengue Fever Vector, *Aedes Aegypti*." *Climatic Change* 48 (2-3): 441-63. doi:10.1023/A:1010717502442.

Hunsperger, Elizabeth A., Sutee Yoksan, Philippe Buchy, Vinh Chau Nguyen, Shamala D. Sekaran, Delia A. Enria, Jose L. Pelegrino, et al. 2009. "Evaluation of Commercially Available Anti-Dengue Virus Immunoglobulin M Tests." *Emerging Infectious Diseases* 15 (3): 436-40. doi:10.3201/eid1503.080923.

Hunter, Paul R., and R. C. Andrew Thompson. 2005. "The Zoonotic Transmission of Giardia and Cryptosporidium." *International Journal for Parasitology, Parasitic Zoonoses - Emerging Issues*, 35 (11–12): 1181–90. doi:10.1016/j.ijpara.2005.07.009.

Innis, B. L., A. Nisalak, S. Nimmannitya, S. Kusalerdchariya, V. Chongswasdi, S. Suntayakorn, P. Puttisri, and C. H. Hoke. 1989. "An Enzyme-Linked Immunosorbent Assay to Characterize Dengue Infections Where Dengue and Japanese Encephalitis Co-Circulate." *The American Journal of Tropical Medicine and Hygiene* 40 (4): 418–27.

Jeneby, Maamun M, M A Suleman, and C Gichuki. 2002. "Sero-Epizootiologic Survey of Trypanosoma Brucei in Kenyan Nonhuman Primates." *Journal of Zoo and Wildlife Medicine: Official Publication of the American Association of Zoo Veterinarians* 33 (4): 337–41.

Johnson, Alison J., Denise A. Martin, Nick Karabatsos, and John T. Roehrig. 2000. "Detection of Anti-Arboviral Immunoglobulin G by Using a Monoclonal Antibody-Based Capture Enzyme-Linked Immunosorbent Assay." *Journal of Clinical Microbiology* 38 (5): 1827–31.

Johnson, Barbara W., Brandy J. Russell, and Robert S. Lanciotti. 2005. "Serotype-Specific Detection of Dengue Viruses in a Fourplex Real-Time Reverse Transcriptase PCR Assay." *Journal of Clinical Microbiology* 43 (10): 4977–83. doi:10.1128/JCM.43.10.4977-4983.2005.

Johnson, B. K., D. Ocheng, A. Gichogo, M. Okiro, D. Libondo, P. Kinyanjui, and P. M. Tukei. 1982. "Epidemic Dengue Fever Caused by Dengue Type 2 Virus in Kenya: Preliminary Results of Human Virological and Serological Studies." *East African Medical Journal* 59 (12): 781–84.

Joshi, Vinod, D. T. Mourya, and R. C. Sharma. 2002. "Persistence of Dengue-3 Virus through Transovarial Transmission Passage in Successive Generations of Aedes Aegypti Mosquitoes." *The American Journal of Tropical Medicine and Hygiene* 67 (2): 158–61.

Karesh, William B., Robert A. Cook, Elizabeth L. Bennett, and James Newcomb. 2005. "Wildlife Trade and Global Disease Emergence." *Emerging Infectious Diseases* 11 (7): 1000–1002. doi:10.3201/eid1107.050194.

Klungthong, Chonticha, Robert V. Gibbons, Butsaya Thaisomboonsuk, Ananda Nisalak, Siripen Kalayanarooj, Vipra Thirawuth, Naowayubol Nutkumhang, Mammen P. Mammen, and Richard G. Jarman. 2007. "Dengue Virus Detection Using Whole Blood for Reverse Transcriptase PCR and Virus Isolation." *Journal of Clinical Microbiology* 45 (8): 2480–85. doi:10.1128/JCM.00305-07.

Kuhn, Richard J, Wei Zhang, Michael G Rossmann, Sergei V Pletnev, Jeroen Corver, Edith Lenches, Christopher T Jones, et al. 2002. "Structure of Dengue Virus: Implications for Flavivirus Organization, Maturation, and Fusion." *Cell* 108 (5): 717–25.

Kuno, G., I. Gómez, and D. J. Gubler. 1991. "An ELISA Procedure for the Diagnosis of Dengue Infections." *Journal of Virological Methods* 33 (1–2): 101–13. doi:10.1016/0166-0934(91)90011-N.

Lanciotti, R S, C H Calisher, D J Gubler, G J Chang, and A V Vorndam. 1992. "Rapid Detection and Typing of Dengue Viruses from Clinical Samples by Using Reverse Transcriptase-Polymerase Chain Reaction." *Journal of Clinical Microbiology* 30 (3): 545–51.

Levi, Tal, Eitan Ben-Dov, Preeti Shahi, Dov Borovsky, and Arie Zaritsky. 2014. "Growth and Development of *Aedes Aegypti* Larvae at Limiting Food Concentrations." *Acta Tropica* 133 (May): 42–44. doi:10.1016/j.actatropica.2014.02.001.

Li, Long, Shee-Mei Lok, I-Mei Yu, Ying Zhang, Richard J Kuhn, Jue Chen, and Michael G Rossmann. 2008. "The Flavivirus Precursor Membrane-Envelope Protein Complex: Structure and Maturation." *Science (New York, N.Y.)* 319 (5871): 1830–34. doi:10.1126/science.1153263.

Low, Jenny G. H., Adrian Ong, Li Kiang Tan, Shera Chaterji, Angelia Chow, Wen Yan Lim, Koon Wui Lee, et al. 2011. "The Early Clinical Features of Dengue in Adults: Challenges for Early Clinical Diagnosis." *PLoS Negl Trop Dis* 5 (5): e1191. doi:10.1371/journal.pntd.0001191.

Lutomiah, Joel, Joshua Bast, Jeffrey Clark, Jason Richardson, Santos Yalwala, David Oullo, James Mutisya, et al. 2013. "Abundance, Diversity, and Distribution of Mosquito Vectors in Selected Ecological Regions of Kenya: Public Health Implications." *Journal of Vector Ecology* 38 (1): 134–42. doi:10.1111/j.1948-7134.2013.12019.x.

Mahmood, Shahid, Hiba Nabeel, Saadia Hafeez, Urooj Zahra, and Hammad Nazeer. 2013. "Seroprevalence of Dengue IgG Antibodies among Healthy Adult Population in Lahore, Pakistan." *ISRN Tropical Medicine* 2013: 1–6. doi:10.1155/2013/521396.

Malavige, G N, S Fernando, D J Fernando, and S L Seneviratne. 2004. "Dengue Viral Infections." *Postgraduate Medical Journal* 80 (948): 588–601. doi:10.1136/pgmj.2004.019638.

Martina, Byron E. E., Penelope Koraka, and Albert D. M. E. Osterhaus. 2009a. "Dengue Virus Pathogenesis: An Integrated View." *Clinical Microbiology Reviews* 22 (4): 564–81. doi:10.1128/CMR.00035-09.

Martin, Denise A., Brad J. Biggerstaff, Becky Allen, Alison J. Johnson, Robert S. Lanciotti, and John T. Roehrig. 2002. "Use of Immunoglobulin M Cross-Reactions in Differential Diagnosis of Human Flaviviral Encephalitis Infections in the United States." *Clinical and Diagnostic Laboratory Immunology* 9 (3): 544–49. doi:10.1128/CDLI.9.3.544-549.2002.

Martin, Denise A., David A. Muth, Teresa Brown, Alison J. Johnson, Nick Karabatsos, and John T. Roehrig. 2000. "Standardization of Immunoglobulin M Capture Enzyme-Linked Immunosorbent Assays for Routine Diagnosis of Arboviral Infections." *Journal of Clinical Microbiology* 38 (5): 1823–26.

McMichael, A J. 2004. "Environmental and Social Influences on Emerging Infectious Diseases: Past, Present and Future." *Philosophical Transactions of the Royal Society B: Biological Sciences* 359 (1447): 1049–58. doi:10.1098/rstb.2004.1480.

Mercado-Curiel, Ricardo F, Hector Armando Esquinca-Aviles, Rosalinda Tovar, Alvaro Diaz-Badillo, Minerva Camacho-Nuez, and Maria de Lourdes Munoz. 2006. "The Four Serotypes of Dengue Recognize the Same Putative Receptors in *Aedes Aegypti* Midgut and *Ae. Albopictus* Cells." *BMC Microbiology* 6 (October): 85. doi:10.1186/1471-2180-6-85.

Metselaar, D., C. R. Grainger, K. G. Oei, D. G. Reynolds, M. Pudney, C. J. Leake, P. M. Tukei, R. M. D'Offay, and D. I. H. Simpson. 1980. "An Outbreak of Type 2 Dengue Fever in the Seychelles, Probably Transmitted by *Aedes Albopictus* (Skuse)." *Bulletin of the World Health Organization* 58 (6): 937–43.

Michalak, K., C. Austin, S. Diesel, M. J. Bacon, P. Zimmerman, and J. N. Maslow. 1998. "Mycobacterium Tuberculosis Infection as a Zoonotic Disease: Transmission between Humans and Elephants." *Emerging Infectious Diseases* 4 (2): 283–87.

Modis, Yorgo, Steven Ogata, David Clements, and Stephen C Harrison. 2003. "A Ligand-Binding Pocket in the Dengue Virus Envelope Glycoprotein." *Proceedings of the National Academy of Sciences of the United States of America* 100 (12): 6986–91. doi:10.1073/pnas.0832193100.

Monath, T P. 1994. "Dengue: The Risk to Developed and Developing Countries." *Proceedings of the National Academy of Sciences of the United States of America* 91 (7): 2395–2400.

Mukhopadhyay, Suchetana, Richard J. Kuhn, and Michael G. Rossmann. 2005. "A Structural Perspective of the Flavivirus Life Cycle." *Nature Reviews Microbiology* 3 (1): 13–22. doi:10.1038/nrmicro1067.

Murphy, Brian R., and Stephen S. Whitehead. 2011. "Immune Response to Dengue Virus and Prospects for a Vaccine *." *Annual Review of Immunology* 29 (1): 587–619. doi:10.1146/annurev-immunol-031210-101315.

Nakgoi, Khajornpong, Narong Nitatpattana, Worawidh Wajjwalku, Pornsawan Pongsopawijit, Supakarn Kaewchot, Sutee Yoksan, Voravit Siripolwat, Marc Souris, and Jean-Paul Gonzalez. 2014. "Dengue, Japanese Encephalitis and Chikungunya Virus Antibody Prevalence among Captive Monkey (*Macaca Nemestrina*) Colonies of Northern

Thailand.” *American Journal of Primatology* 76 (1): 97–102. doi:10.1002/ajp.22213.

Olson, Sarah H., Ronald Gangnon, Guilherme Abbad Silveira, and Jonathan A. Patz. 2010. “Deforestation and Malaria in M?ncio Lima County, Brazil.” *Emerging Infectious Diseases* 16 (7): 1108–15. doi:10.3201/eid1607.091785.

Pagès, Frédéric, Christophe N Peyrefitte, Médard Toung Mve, Fanny Jarjaval, Sylvain Brisse, Isabelle Iteman, Patrick Gravier, Hugues Tolou, Dieudonné Nkoghe, and Marc Grandadam. 2009. “Aedes Albopictus Mosquito: The Main Vector of the 2007 Chikungunya Outbreak in Gabon.” *PloS One* 4 (3): e4691. doi:10.1371/journal.pone.0004691.

Palucka, Anna Karolina. 2000. “Dengue Virus and Dendritic Cells.” *Nature Medicine* 6 (7): 748–49. doi:10.1038/77470.

Pancharoen, C, and U Thisyakorn. 2001. “Neurological Manifestations in Dengue Patients.” *The Southeast Asian Journal of Tropical Medicine and Public Health* 32 (2): 341–45.

Parrish, Colin R., Edward C. Holmes, David M. Morens, Eun-Chung Park, Donald S. Burke, Charles H. Calisher, Catherine A. Laughlin, Linda J. Saif, and Peter Daszak. 2008. “Cross-Species Virus Transmission and the Emergence of New Epidemic Diseases.” *Microbiology and Molecular Biology Reviews* 72 (3): 457–70. doi:10.1128/MMBR.00004-08.

Patel, Biren A., and Roshna E. Wunderlich. 2010. “Dynamic Pressure Patterns in the Hands of Olive Baboons (*Papio Anubis*) During Terrestrial Locomotion: Implications for Cercopithecoid Primate Hand Morphology.” *The Anatomical Record: Advances in Integrative Anatomy and Evolutionary Biology* 293 (4): 710–18. doi:10.1002/ar.21128.

Peeling, Rosanna W., Harvey Artsob, Jose Luis Pelegrino, Philippe Buchy, Mary J. Cardoso, Shamala Devi, Delia A. Enria, et al. 2010. “Evaluation of Diagnostic Tests: Dengue.” *Nature Reviews Microbiology* 8 (December): S30–37. doi:10.1038/nrmicro2459.

Prado, Irina, Delfina Rosario, Lídice Bernardo, Mayling Álvarez, Rosmari Rodríguez, Susana Vázquez, and María G. Guzmán. 2005. “PCR Detection of Dengue Virus Using Dried Whole Blood Spotted on Filter Paper.” *Journal of Virological Methods* 125 (1): 75–81. doi:10.1016/j.jviromet.2005.01.001.

Puccioni-Sohler, Marzia, Marco Orsini, and Cristiane N. Soares. 2012. “Dengue: A New Challenge for Neurology.” *Neurology International* 4 (3). doi:10.4081/ni.2012.e15.

Quintero, Juliana, Helena Brochero, Pablo Manrique-Saide, Mario Barrera-Perez, Cesar Basso, Sonnia Romero, Andrea Caprara, et al. 2014. “Ecological, Biological and Social Dimensions of Dengue Vector Breeding in Five Urban Settings of Latin

America: A Multi-Country Study." *BMC Infectious Diseases* 14 (January): 38.
doi:10.1186/1471-2334-14-38.

Ranjit, Suchitra, and Niranjan Kissoon. 2011. "Dengue Hemorrhagic Fever and Shock Syndromes." *Pediatric Critical Care Medicine: A Journal of the Society of Critical Care Medicine and the World Federation of Pediatric Intensive and Critical Care Societies* 12 (1): 90–100. doi:10.1097/PCC.0b013e3181e911a7.

Rao, Sachin, Manish Kumar, Soumik Ghosh, and Adesh Kumar Gadpayle. 2013. "A Rare Case of Dengue Encephalitis." *BMJ Case Reports* 2013. doi:10.1136/bcr-2012-008229.

Reiter, P. 2001. "Climate Change and Mosquito-Borne Disease." *Environmental Health Perspectives* 109 (Suppl 1): 141–61.

Rodenhuis-Zybert, Izabela A., Jan Wilschut, and Jolanda M. Smit. 2010. "Dengue Virus Life Cycle: Viral and Host Factors Modulating Infectivity." *Cellular and Molecular Life Sciences* 67 (16): 2773–86. doi:10.1007/s00018-010-0357-z.

Rohani, A., I. Zamree, R. T. Joseph, and H. L. Lee. 2008. "Persistency of Transovarial Dengue Virus in *Aedes Aegypti* (Linn.)." *The Southeast Asian Journal of Tropical Medicine and Public Health* 39 (5): 813–16.

Rothman, Alan L. 2010. "Cellular Immunology of Sequential Dengue Virus Infection and Its Role in Disease Pathogenesis." *Current Topics in Microbiology and Immunology* 338: 83–98. doi:10.1007/978-3-642-02215-9_7.

Rubens Costa Lima, José, Maria Zélia Rouquayrol, Maria Roseli Monteiro Callado, Maria Izabel Florindo Guedes, and Claudia Pessoa. 2012. "Interpretation of the Presence of IgM and IgG Antibodies in a Rapid Test for Dengue: Analysis of Dengue Antibody Prevalence in Fortaleza City in the 20th Year of the Epidemic." *Revista Da Sociedade Brasileira de Medicina Tropical* 45 (2): 163–67.

Salazar, Ma Isabel, Jason H. Richardson, Irma Sánchez-Vargas, Ken E. Olson, and Barry J. Beaty. 2007. "Dengue Virus Type 2: Replication and Tropisms in Orally Infected *Aedes Aegypti* Mosquitoes." *BMC Microbiology* 7 (1): 9. doi:10.1186/1471-2180-7-9.

Sirivichayakul, Chukiat, Kriengsak Limkittikul, Pornthep Chanthavanich, Vithaya Jiwariyavej, Watcharee Chokejindachai, Krisana Pengsaa, Saravudh Suvannadabba, Wut Dulyachai, G. William Letson, and Arunee Sabchareon. 2012. "Dengue Infection in Children in Ratchaburi, Thailand: A Cohort Study. II. Clinical Manifestations." *PLoS Neglected Tropical Diseases* 6 (2).
doi:10.1371/journal.pntd.0001520.

Smith, C E G. 1956. "The History of Dengue in Tropical Asia and Its Probable

Relationship to the Mosquito *Aedes Aegypti*." *The Journal of Tropical Medicine and Hygiene* 59 (10): 243–51.

Solomon, T, N M Dung, D W Vaughn, R Kneen, L T Thao, B Raengsakulrach, H T Loan, et al. 2000. "Neurological Manifestations of Dengue Infection." *Lancet* 355 (9209): 1053–59. doi:10.1016/S0140-6736(00)02036-5.

Stephens, H A F. 2010. "HLA and Other Gene Associations with Dengue Disease Severity." *Current Topics in Microbiology and Immunology* 338: 99–114. doi:10.1007/978-3-642-02215-9_8.

Streicker, Daniel G., Amy S. Turmelle, Maarten J. Vonhof, Ivan V. Kuzmin, Gary F. McCracken, and Charles E. Rupprecht. 2010. "Host Phylogeny Constrains Cross-Species Emergence and Establishment of Rabies Virus in Bats." *Science* 329 (5992): 676–79. doi:10.1126/science.1188836.

Suleman. 1999. *Peripheral Blood Lymphocyte Immunocompetence in Wild African Green Monkeys (Cercopithecus Aethiops) and the Effects of Capture and Confinement*. Vol. 13. 1 vols. In vivo.

Tabachnick, Walter J., Leonard E. Munstermann, and Jeffrey R. Powell. 1979. "Genetic Distinctness of Sympatric Forms of *Aedes Aegypti* in East Africa." *Evolution* 33 (1): 287. doi:10.2307/2407619.

Taylor, L H, S M Latham, and M E Woolhouse. 2001. "Risk Factors for Human Disease Emergence." *Philosophical Transactions of the Royal Society B: Biological Sciences* 356 (1411): 983–89. doi:10.1098/rstb.2001.0888.

Tei, Shuchin, Naoto Kitajima, Kazuaki Takahashi, and Shunji Mishiro. 2003. "Zoonotic Transmission of Hepatitis E Virus from Deer to Human Beings." *The Lancet* 362 (9381): 371–73. doi:10.1016/S0140-6736(03)14025-1.

Teoh, Boon-Teong, Sing-Sin Sam, Juraina Abd-Jamil, and Sazaly AbuBakar. 2010. "Isolation of Ancestral Sylvatic Dengue Virus Type 1, Malaysia." *Emerging Infectious Diseases* 16 (11): 1783–85. doi:10.3201/eid1611.100721.

Thongrungrat, Supatra, Ladawan Wasinpiyamongkol, Pannamas Maneekan, Samrerng Prummongkol, and Yudthana Samung. 2012. "Natural Transovarial Dengue Virus Infection Rate in Both Sexes of Dark and Pale Forms of *Aedes Aegypti* from an Urban Area of Bangkok, Thailand." *The Southeast Asian Journal of Tropical Medicine and Public Health* 43 (5): 1146–52.

Tipayamongkhogul, Mathuros, Chi-Tai Fang, Suratsawadee Klinchan, Chung-Ming Liu, and Chwan-Chuen King. 2009. "Effects of the El Niño-Southern Oscillation on Dengue Epidemics in Thailand, 1996-2005." *BMC Public Health* 9 (1): 422. doi:10.1186/1471-2458-9-422.

Ukey, PM, SA Bondade, PV Paunipagar, RM Powar, and SL Akulwar. 2010. "Study of Seroprevalence of Dengue Fever in Central India." *Indian Journal of Community Medicine : Official Publication of Indian Association of Preventive & Social Medicine* 35 (4): 517–19. doi:10.4103/0970-0218.74366.

Vasilakis, Nikos, Jane Cardoso, Mawlouth Diallo, Amadou A Sall, Edward C Holmes, Kathryn A Hanley, Scott C Weaver, Javier Mota, and Rebeca Rico-Hesse. 2010. "Sylvatic Dengue Viruses Share the Pathogenic Potential of Urban/endemic Dengue Viruses." *Journal of Virology* 84 (7): 3726–27; author reply 3727–28. doi:10.1128/JVI.02640-09.

Vasilakis, Nikos, Jane Cardoso, Kathryn A. Hanley, Edward C. Holmes, and Scott C. Weaver. 2011. "Fever from the Forest: Prospects for the Continued Emergence of Sylvatic Dengue Virus and Its Impact on Public Health." *Nature Reviews Microbiology* 9 (7): 532–41. doi:10.1038/nrmicro2595.

Vasilakis, Nikos, Eleanor R. Deardorff, Joan L. Kenney, Shannan L. Rossi, Kathryn A. Hanley, and Scott C. Weaver. 2009. "Mosquitoes Put the Brake on Arbovirus Evolution: Experimental Evolution Reveals Slower Mutation Accumulation in Mosquito Than Vertebrate Cells." *PLoS Pathogens* 5 (6). doi:10.1371/journal.ppat.1000467.

Vasilakis, Nikos, Edward C. Holmes, Eric B. Fokam, Ousmane Faye, Mawlouth Diallo, Amadou A. Sall, and Scott C. Weaver. 2007. "Evolutionary Processes among Sylvatic Dengue Type 2 Viruses." *Journal of Virology* 81 (17): 9591–95. doi:10.1128/JVI.02776-06.

Vasilakis, Nikos, Elisabeth J Shell, Eric B Fokam, Peter W Mason, Kathryn A Hanley, D Mark Estes, and Scott C Weaver. 2007. "Potential of Ancestral Sylvatic Dengue-2 Viruses to Re-Emerge." *Virology* 358 (2): 402–12. doi:10.1016/j.virol.2006.08.049.

Vasilakis, Nikos, Robert B Tesh, and Scott C Weaver. 2008a. "Sylvatic Dengue Virus Type 2 Activity in Humans, Nigeria, 1966." *Emerging Infectious Diseases* 14 (3): 502–4. doi:10.3201/eid1403.070843.

Vasilakis, Nikos, and Scott C Weaver. 2008. "The History and Evolution of Human Dengue Emergence." *Advances in Virus Research* 72: 1–76. doi:10.1016/S0065-3527(08)00401-6.

Volney, Béatrice, Jean-François Pouliquen, Benoît De Thoisy, and Thierry Fandeur. 2002. "A Sero-Epidemiological Study of Malaria in Human and Monkey Populations in French Guiana." *Acta Tropica* 82 (1): 11–23.

Wang, Eryu, Haolin Ni, Renling Xu, Alan D. T. Barrett, Stanley J. Watowich, Duane J. Gubler, and Scott C. Weaver. 2000. "Evolutionary Relationships of

Endemic/Epidemic and Sylvatic Dengue Viruses." *Journal of Virology* 74 (7): 3227–34. doi:10.1128/JVI.74.7.3227-3234.2000.

Weaver, Scott C, and William K Reisen. 2010. "Present and Future Arboviral Threats." *Antiviral Research* 85 (2): 328–45. doi:10.1016/j.antiviral.2009.10.008.

Were, Fred. 2012. "The Dengue Situation in Africa." *Paediatrics and International Child Health* 32 (s1): 18–21. doi:10.1179/2046904712Z.000000000048.

Whitehead, Stephen S., Joseph E. Blaney, Anna P. Durbin, and Brian R. Murphy. 2007. "Prospects for a Dengue Virus Vaccine." *Nature Reviews Microbiology* 5 (7): 518–28. doi:10.1038/nrmicro1690.

"WHO | Dengue and Severe Dengue." 2013a. WHO. Accessed April 12. <http://www.who.int/mediacentre/factsheets/fs117/en/>.

"WHO | Dengue Vaccine Research." 2013. WHO. Accessed July 3. http://www.who.int/vaccine_research/diseases/dengue/dengue_vaccines/en/.

Wichit, Sineewanlaya, Akanitt Jittmittraphap, Kazuya I P J Hidari, Butsaya Thaisomboonsuk, Songsak Petmitr, Sukathida Ubol, Chie Aoki, et al. 2011. "Dengue Virus Type 2 Recognizes the Carbohydrate Moiety of Neutral Glycosphingolipids in Mammalian and Mosquito Cells." *Microbiology and Immunology* 55 (2): 135–40. doi:10.1111/j.1348-0421.2010.00293.x.

Wilder-Smith, Annelies, and Eli Schwartz. 2005. "Dengue in Travelers." *The New England Journal of Medicine* 353 (9): 924–32. doi:10.1056/NEJMra041927.

Wolfe, N D, A M Kilbourn, W B Karesh, H A Rahman, E J Bosi, B C Cropp, M Andau, A Spielman, and D J Gubler. 2001. "Sylvatic Transmission of Arboviruses among Bornean Orangutans." *The American Journal of Tropical Medicine and Hygiene* 64 (5-6): 310–16.

Wu, S J, G Grouard-Vogel, W Sun, J R Mascola, E Brachtel, R Putvatana, M K Louder, et al. 2000. "Human Skin Langerhans Cells Are Targets of Dengue Virus Infection." *Nature Medicine* 6 (7): 816–20. doi:10.1038/77553.

Zanotto, P. M., E. A. Gould, G. F. Gao, P. H. Harvey, and E. C. Holmes. 1996. "Population Dynamics of Flaviviruses Revealed by Molecular Phylogenies." *Proceedings of the National Academy of Sciences* 93 (2): 548–53.

Zhang, Wei, Paul R Chipman, Jeroen Corver, Peter R Johnson, Ying Zhang, Suchetana Mukhopadhyay, Timothy S Baker, James H Strauss, Michael G Rossmann, and Richard J Kuhn. 2003. "Visualization of Membrane Protein Domains by Cryo-Electron Microscopy of Dengue Virus." *Nature Structural Biology* 10 (11): 907–12. doi:10.1038/nsb990.

APPENDICES

Appendix 1

Reagents for IgG Antibody ELISA

1. Coating Buffer (1X PBS)

NaCl	8g
KCl	0.2g
KH ₂ PO ₂	0.14g
Na ₂ HPO ₂	0.91g

Top with distilled water to 1L. pH to 7.4

2. Blocking Buffer

5% of skimmed milk in coating buffer

3. Phosphate Citrate Buffer

Citric acid	0.47g
Na ₂ HPO ₄	0.73g

Top with distilled water to 100ml. pH to 5.0

4. Substrate Solution

Phosphate buffer	25ml
OPD	10mg
Hydrogen Peroxide	10μl

IgG antibody ELISA template

Date: _____

Assay: _____

Samples: _____

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank											
B	Blank											
C	Negative Control											
D	Negative Control											
E	Positive Control											
F	Positive Control											
G												
H												

Sample Dilution: _____

Monoclonal Antibody Dilution: _____

Conjugate Dilution: _____

Incubation time Time in Time out Temperature

1				
2				
3				
4				

Notes:

1	
2	
3	
4	
5	

Appendix 2

Reagents for RT-PCR-Amplification of Dengue Virus

Starting from dsRNA:

dsRNA Samples used:

Extraction Methods used: RevertAid Premium First Strand cDNA Synthesis kit
(#K1651, #K1652)

Reverse Transcription

Master Mix Preparation

Reagents	1XReaction	_____ X Reaction
dd H ₂ O Nuclease free	8µl	_____
10mM dNTP's	1 µl	_____
Random Hexamar	1 µl	_____
Template RNA (each sample)	5 µl	

Temperatures:

Incubate at 65°C for 5 minutes

Reagents	1XReaction	_____ X Reaction
5X RT Buffer	4µl	_____
Revert Aid Premium Enzyme Mix	1µl	_____

Temperatures:

Incubate at: 25°C for 10 minutes
50°C for 30 minutes
85°C for 5 minutes

i) PCR Amplification of cDNA

cDNA used:

Primers: D1 forward and D2 Reverse

Reagents	1X Reaction	_____X Reaction
ddH ₂ O Nuclease free	22 μ l	_____X Reaction
Dream Taq Master mix (2X)	25 μ l	_____X Reaction
Forward Primer	1 μ l	_____X Reaction
Reverse Primer	1 μ l	_____X Reaction
Template DNA	1 μ l	_____X Reaction

Amplification

File number:

Cycles: 30

Temperatures: 30 sec-94°C (Denaturation of DNA), 1 Min-55°C (Hybridization of primers DNA), 2 Min-72°C (Chain Elongation), 5 Min-72°C (Final Extension)

ii) Re-amplification of cDNA

cDNA used:

Primers: D1 forward and TS2 reverse.

Reagents are as above except for the primers.

Amplification

File number:

Cycles: 26

Temperatures: 30 sec- 94°C, 1min-55°C, 2min-72°C.

REAGENTS (PCR Continued)

1. 2% Agarose gel (100ml)

2g of agarose powder in 100ml of 1 x TAE buffer (pH 7.9). Heat to dissolve. Check the volume then top it up to 100ml with distilled water if it had evaporated. Cool the solution then add 5 μ l of ethidium bromide. Swirl then cast the gel into the tank and allow for polymerisation to take place.

2. Ethidium Bromide

Caution: Ethidium bromide (EtBr) is a powerful mutagen and is moderately toxic. Wear gloves when working with solutions containing this dye. To prepare add 10mg of EtBr to 1ml dH₂O or dissolve 1 x 100mg EtBr Tablet in 10ml dH₂O. Store the solution in a dark bottle because it is light sensitive.

3. a) 10x Tris Acetate EDTA Buffer (TAE Buffer)

0.4M Tris base	121g
0.01M Glacial Acetic Acid	28.58ml
0.5M EDTA pH 8.0	50ml

Dissolve in dH₂O and adjust pH to 7.9 with NaCl. Top up with distilled water to 2.5 mls for the final volume.

b) 1x TAE (pH 7.9)

To make one litre take 100ml of the 10XTAE and top up to 1000ml dH₂O.

4. Molecular Weight Markers\GeneRuler (Thermo scientific)

Thermo Scientific GeneRuler 100bp Plus DNA Ladder is designed for sizing and approximate quantification of wide range double-stranded DNA on agarose and polyacrylamide gels. The ladder is composed of fourteen chromatography-

purified individual DNA fragments (in base pairs): 3000, 2000, 1500, 1200, **1000**, 900, 800, 700, 600, **500**, 400, 300, 200, 100. It contains two reference bands (1000 and 500 bp) for easy orientation.

The ladder is dissolved in TE buffer.

2% Agarose gels were electrophoresed at 100V.

Appendix 3

DENV 2 Genotyping

PCR Protocol:

- Step 1: RT-PCR of general dengue genes using primers D1 and D2.
(30 cycles: 94°C - 30 seconds, 55°C- 1 Minutes, 72°C-2 minutes, 72°C-5 Minutes)
- Step 2: Re-amplification of the step 1 amplicones was done using type specific primers. i.e D1 and TS2.
(26 cycles: 30 seconds- 94°C, 1 minute-55°C, 2 minutes-72°C).

Primer Sequences

Primers to be used in this study are upstream dengue virus consensus primer (D1) 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' and downstream consensus primer (D2) 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3' (Lanciotti *et. al.*, 1992, Prado *et. al.*, 2005).

The type specific primers to be used on nested PCR is upstream DENV 2 (D1) 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' and downstream specific primer TS1 5'-CGCCACAAGGGCCATGAACAG-3'.

Primer	Sequence	Genome Position	Size in Bp of DNA Amplicons
D1	5'- TCAATATGCTGAAACGCGCGAGAAACCG- 3'	134-161	511
D2	5'- TTGCACCAACAGTCAATGTCTTCAGGTTC -3'	616-644	511
TS1	5'-CGCCACAAGGGCCATGAACAG-3'.	232-252	119 (D1 and TS1)

Appendix 4

Picture of Olive Baboons In The Wild



Appendix 5

Picture of an African Green Monkey in the Wild

