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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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 Mutuiri, 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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<th>Definition</th>
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<tbody>
<tr>
<td>1X</td>
<td>One times the normal concentration</td>
</tr>
<tr>
<td>A</td>
<td>Aedes</td>
</tr>
<tr>
<td>Ae</td>
<td>Aedes</td>
</tr>
<tr>
<td>AC</td>
<td>Alternating Current</td>
</tr>
<tr>
<td>AGM</td>
<td>African green monkey</td>
</tr>
<tr>
<td>C</td>
<td>Capsid</td>
</tr>
<tr>
<td>C. aethiops</td>
<td>Chlorocebus aethiops</td>
</tr>
<tr>
<td>cDNA</td>
<td>Copy deoxyribonucleic acid</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>Dendritic cell specific intracellular adhesion molecules grabbing intergrin</td>
</tr>
<tr>
<td>DENV</td>
<td>Dengue virus</td>
</tr>
<tr>
<td>DENV 1</td>
<td>Dengue virus 1</td>
</tr>
<tr>
<td>DENV 1-4</td>
<td>Dengue virus 1-4</td>
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<tr>
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<td>Dengue virus 2</td>
</tr>
<tr>
<td>DENV 4</td>
<td>Dengue virus 4</td>
</tr>
<tr>
<td>DHF</td>
<td>Dengue hemorrhagic fever</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>GAGs</td>
<td>Glycosaminoglycans</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesive molecule</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin gamma</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>NHPs</td>
<td>Non-human primates</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometer</td>
</tr>
<tr>
<td>NS</td>
<td>Non-structural proteins</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OPD</td>
<td>Orthophenylene diamine</td>
</tr>
<tr>
<td>P. anubis</td>
<td>Papio anubis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS\Tween</td>
<td>PBS containing Tween 20</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA buffer</td>
</tr>
<tr>
<td>TS2</td>
<td>Type specific primer to dengue 2</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
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</table>
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 molecular 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 sera-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).
Mating

Adult emerges from the pupa

Pupa

Fourth larval stage

Third larval stage

Larva

Imago

Egg

Oviposition, most times slightly above the water surface

Eggs develop to larvae in the water

Femaleneeds blood for the production of eggs

Moultinebetween each stage

First larval stage

Second larval stage

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 1μl 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 (Thongrungkhiat *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
Transovarial transmission also provides a mechanism to allow DENV to survive dry or cold seasons or the temporary absence of non-immune vertebrate hosts (Thongrungkiat 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 nucleo-capsid 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.thescientist.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).

![Diagram of E protein domains](image)

**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 immune-chromographic 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 fetal. 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/mm³) (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 simine (B virus)- Rhesus monkey, Monkey pox- *Cercopithecus ascanis*, *C. nigrovidis*, *C. petauria*, Chikungunya- *Papio ursinos*, *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 formite (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.

\[
N = \frac{Z^2 \cdot P(1-P)}{D^2}
\]

**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™ 11C 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: 8 μl 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 RevertAidTM 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 22 μl 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, Norwlk, 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 transluminator, 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 transluminator, 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 ($\chi^2$) 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

<table>
<thead>
<tr>
<th>Location</th>
<th>No. of Animals Tested</th>
<th>No. Positive for Antibody ELISA</th>
<th>Percentage (%) Positive for Antibody ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aberdare</td>
<td>32</td>
<td>17</td>
<td>53.1%</td>
</tr>
<tr>
<td>Kajiado</td>
<td>25</td>
<td>8</td>
<td>32%</td>
</tr>
<tr>
<td>Kakamega</td>
<td>20</td>
<td>8</td>
<td>40%</td>
</tr>
<tr>
<td>Kibwezi</td>
<td>23</td>
<td>10</td>
<td>43.5</td>
</tr>
<tr>
<td>Kitale</td>
<td>19</td>
<td>9</td>
<td>47.4</td>
</tr>
<tr>
<td>Laikipia</td>
<td>115</td>
<td>41</td>
<td>35.7</td>
</tr>
<tr>
<td>Nairobi</td>
<td>28</td>
<td>8</td>
<td>28.6</td>
</tr>
<tr>
<td>Namanga</td>
<td>25</td>
<td>8</td>
<td>32%</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>287</strong></td>
<td><strong>109</strong></td>
<td><strong>37.98%</strong></td>
</tr>
</tbody>
</table>
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, α <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, α < 0.05).
Table 4.2: Sero prevalence of dengue antibodies comparing regions, age, gender and species exposure rates.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
<th>Number Examined (n)</th>
<th>Positive by IgG ELISA</th>
<th>Positive (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regions</td>
<td>Low-lands</td>
<td>73</td>
<td>26</td>
<td>35.6</td>
<td>0.677</td>
</tr>
<tr>
<td></td>
<td>High-lands</td>
<td>214</td>
<td>83</td>
<td>38.8</td>
<td></td>
</tr>
<tr>
<td>Species</td>
<td><em>P. anubis</em></td>
<td>115</td>
<td>41</td>
<td>35.7</td>
<td>0.537</td>
</tr>
<tr>
<td></td>
<td><em>C. aethiopes</em></td>
<td>172</td>
<td>68</td>
<td>39.5</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Adult</td>
<td>159</td>
<td>67</td>
<td>42.1</td>
<td>0.113</td>
</tr>
<tr>
<td></td>
<td>Juvenile</td>
<td>128</td>
<td>42</td>
<td>32.8</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>127</td>
<td>48</td>
<td>37.8</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>160</td>
<td>61</td>
<td>38.1</td>
<td></td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>Species</th>
<th>Location</th>
<th>Positive by RT-PCR</th>
<th>Positive Percentage (%)</th>
<th>Negative Percentage (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. anubis</em></td>
<td>Laikipia</td>
<td>2</td>
<td>4.88</td>
<td>95.12</td>
<td>1.000</td>
</tr>
<tr>
<td><em>C. aethiops</em></td>
<td>Kakamega</td>
<td>1</td>
<td>6.25</td>
<td>93.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kibwezi</td>
<td>2</td>
<td>20</td>
<td>80.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kitale</td>
<td>1</td>
<td>11.11</td>
<td>88.89</td>
<td></td>
</tr>
</tbody>
</table>
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 Malaysia 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 (Pages 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
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 aboreal 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 habitant 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


Franco, Leticia, Gustavo Palacios, José Antonio Martínez, 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


APPENDICES

Appendix 1

Reagents for IgG Antibody ELISA

1. Coating Buffer (1X PBS)

   NaCl 8g
   KCl 0.2g
   KH₂PO₂ 0.14g
   Na₂HPO₄ 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
   Na₂HPO₄ 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:** 

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
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<td>C</td>
<td>Negative Control</td>
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</table>

**Sample Dilution:** 

**Monoclonal Antibody Dilution:** 

**Conjugate Dilution:**
<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Time in</th>
<th>Time out</th>
<th>Temperature</th>
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<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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Notes:
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</tr>
<tr>
<td>4</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
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**

<table>
<thead>
<tr>
<th>Reagents</th>
<th>1XReaction</th>
<th>X Reacttion</th>
</tr>
</thead>
<tbody>
<tr>
<td>dd H₂O Nuclease free</td>
<td>8μl</td>
<td></td>
</tr>
<tr>
<td>10mM dNTP's</td>
<td>1 μl</td>
<td></td>
</tr>
<tr>
<td>Random Hexamar</td>
<td>1 μl</td>
<td></td>
</tr>
<tr>
<td>Template RNA (each sample)</td>
<td>5 μl</td>
<td></td>
</tr>
</tbody>
</table>

**Temperatures:**

Incubate at 65°C for 5 minutes

<table>
<thead>
<tr>
<th>Reagents</th>
<th>1XReaction</th>
<th>X Reacttion</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X RT Buffer</td>
<td>4μl</td>
<td></td>
</tr>
<tr>
<td>Revert Aid Premium Enzyme Mix</td>
<td>1μl</td>
<td></td>
</tr>
</tbody>
</table>

**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

<table>
<thead>
<tr>
<th>Reagents</th>
<th>1X Reaction</th>
<th>X Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>ddH₂O Nuclease free</td>
<td>22 µl</td>
<td>X Reaction</td>
</tr>
<tr>
<td>Dream Taq Master mix (2X)</td>
<td>25 µl</td>
<td>X Reaction</td>
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<tr>
<td>Forward Primer</td>
<td>1 µl</td>
<td>X Reaction</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>1 µl</td>
<td>X Reaction</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1 µl</td>
<td>X Reaction</td>
</tr>
</tbody>
</table>

**Amplification**

File number:

Cycles: 30

Temperatures: 30 sec-94°C (Denaturation of DNA), 1 Min-55°C (Hybridation 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 5μl 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 dH$_2$O or dissolve 1 x 100mg EtBr Tablet in 10ml dH$_2$O. 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 dH$_2$O 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 dH$_2$O.

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)

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'.
<table>
<thead>
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<th>Primer</th>
<th>Sequence</th>
<th>Genome Position</th>
<th>Size in Bp of DNA Amplicons</th>
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</thead>
<tbody>
<tr>
<td>D1</td>
<td>5' - TCAATATGCTGAAACGCGCGAGAAACCG-3'</td>
<td>134-161</td>
<td>511</td>
</tr>
<tr>
<td>D2</td>
<td>5' - TTGCACCAACAGTCAATGTCTTCAGGTTTC-3'</td>
<td>616-644</td>
<td>511</td>
</tr>
<tr>
<td>TS1</td>
<td>5' - CGCCACAAGGGCCATGAACAG-3'</td>
<td>232-252</td>
<td>119 (Dl and TS1)</td>
</tr>
</tbody>
</table>
Appendix 4

Picture of Olive Baboons In The Wild
Appendix 5

Picture of an African Green Monkey in the Wild