The Dissertation Committee for Sandra Vanderli Mayer Certifies that this is the approved version of the following dissertation:

DISCOVERY AND CHARACTERIZATION OF A NEWLY EMERGED DENV SEROTYPE

Committee:

Nikos Vasilakis, Ph.D., Supervisor

Alexander J. Indrikovs, M.D.

Ernesto T.A. Marques Jr., M.D., Ph.D.

Robert B. Tesh, M.D.

Gustavo Valbuena, M.D., Ph.D.

Tian Wang, Ph.D.

_____

David W. Niesel, Ph.D.

Dean, Graduate School

DISCOVERY AND CHARACTERIZATION OF A NEWLY EMERGED DENV SEROTYPE

by

Sandra Vanderli Mayer, D.V.M., M.Sc.

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Dedication

To my husband Evandro who kept me motivated and gave me strength, support and love during all the moments of our life.
In memory of my parents Irma Mayer and Hamilton Mayer for the sacrifices they did to allow me to be who I am today. They always will be with me.

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DISCOVERY AND CHARACTERIZATION OF A NEWLY EMERGED DENV SEROTYPE

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Supervisor: Nikos Vasilakis

Dengue virus (DENV) is a mosquito-transmitted flavivirus that presents a substantial threat to public health worldwide. One third of the global population is at risk of infection and over 400 million cases of dengue are reported per year. DENV is maintained in two transmission cycles: a sylvatic cycle between mosquitos and non-human primates (NHP), and an urban cycle between mosquitos and human hosts. The human-endemic lineages of DENV-1-4 each emerged from sylvatic ancestors maintained in a cycle between NHP and arboreal *Aedes* mosquitoes. Ancestral strains are still persisting in both Southeast Asia and West Africa, posing a risk for the contemporary emergence of sylvatic strains into the human population. In this study I describe the discovery and characterization of a novel DENV, isolated from a febrile patient in the Malaysian state of Sarawak, which presumably represents the prototype virus of a new dengue serotype. A complete genetic and serologic analysis was performed to characterize this isolate and to demonstrate that it represents a distinct virus among the other members of the DENV serogroup. The infection was assessed in a NHP model using rhesus macaques to study pathogenesis and homotypic and heterotypic responses to

this novel DENV. A productive infection in NHP was demonstrated, as well the ability of this host to transmit the virus to mosquitoes, which implies the virus can be sustained a transmission cycle in nature. Furthermore, the vector competence of *Aedes aegypti* and *Aedes albopictus* mosquitoes, the two main vectors of human-endemic DENV, was evaluated. The potential of these vectors to be infected and transmit the novel virus was demonstrated. Initial structural studies were performed to determine molecular differences could be present in this novel DENV compared to the other DENV serotypes. Collectively, my study describes the emergence of a novel DENV serotype and its biological characteristics. It also brings new insights to the future development of diagnostics, therapeutics and vaccines.

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List of Abbreviations

Å	Angstrom unit
aa	Amino acid
AAALAC	Association for assessment and accreditation of laboratory animal
AEC	Aminoethylcarbazole
AFRIMS	Armed Forces Research Institute of Medical Sciences
ALT	Alanine aminotransferase
ARC	Animal resources center
AST	Aspartate aminotransferase
ATTC	American type culture collection
AU	Antigenic unit
BASO	Basophils
BP	Blood pressure
C	Capsid (flavivirus-encoded protein)
CBC	Complete blood count
CHIKV	Chikungunya virus
CoV	Coronavirus
CPRC	Caribbean Primate Research Center
CPS	Pentanucleotide sequence
Cryo-EM	Cryo-electron microscopy
DEN	dengue disease
DENRA	Dengue virus recombinant antigen
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DIC	Disseminated intravascular coagulation
DMEM	Dulbecco's minimal essential medium
DMSO	Dimethyl sulfoxide
dpi	Days post-infection
DSS	Dengue shock syndrome
E	Envelope (flavivirus-encoded protein)
EBOV	Ebola virus
EBV	Epstein-Barr virus
ECSA	East-Central-South African
EID	Emerging infectious diseases
EIP	Extrinsic incubation period
ELISA	Enzyme-linked immunosorbent assay
ENSO	El Nino Southern Oscillation
EO	Eosinophils
FBS	fetal bovine serum
FFA	Focus-forming assay
FIA	Focus-forming immunoassay
FIPV	Feline infectious peritonitis virus

FITC	Fluorescein isothiocyanate
GSBS	Graduate School of Biomedical Sciences
HA	Hemagglutinin
HAT	Hypoxanthine-aminopterin-thymidine
HCT	Hematocrit
HGB	Hemoglobin
HIV	Human immunodeficiency virus
HMAF	Hyperimmune mouse ascitic fluid
HRP	Horseradish peroxidase
HS	Heparan sulfate
Huh-7	Human hepatoma (cell line)
IACUC	Institutional Animal Care and Use Committee
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IHC	Immunohistochemistry
IHCM	Institute of Health and Community Medicine
IL	Interleukin
IOL	Indian Ocean Lineage
ISR	Immune Status Ratio
JEV	Japanese encephalitis virus
LDH	Lactase dehydrogenase
LID	Laboratory of Infectious Diseases
LYMPH	Lymphocytes
M	Membrane (flavivirus-encoded protein)
MAbs	Monoclonal antibodies
MCP-1	Monocyte chemotactic protein-1
MEB	Midgut escape barrier
MIAF	Mouse hyperimmune ascitic fluid
MIB	Midgut infectious barrier
ML	Maximum likelihood
moDC	Monocyte-derived dendritic cells
MOI	Multiplicity of infection
MONO	Monocytes
NA	Neuraminidase
NCA	Normal cell antigen
NEUT	Neutrophils
NGS	Next generation sequencing
NHP	Non-human primates
NiV	Nipah virus
NS	Nonstructural
NT	Not tested
OD	Optical density
ORF	Open reading frame
P/S	Penicillin/streptomycin
PAHO	Pan American Health Organization
PBMC	Peripheral blood mononuclear cells

PEG	Polyethylene glycol
pi	Post-infection
PLT	Platelets
POC	Point-of-care
PPE	Personal protection equipment
prM/M	precursor to membrane/membrane (flavivirus-encoded protein)
PRNT	Plaque reduction neutralization test
RBC	Red blood cells
RT	Room temperature
RVFV	Rift valley fever virus
s.c.	subcutaneously
s.c.	Subcutaneously
SARS	Severe acute respiratory syndrome
SARS-CoV	SARS coronavirus
SCID	Severe combined immunodeficiency
SIV	Simian immunodeficiency virus
SL	Stem-loop
SLEV	St. Louis encephalitis
SOP	Standard operation procedure
TBEV	Tick-borne encephalitis virus
TNF-α	Tumor necrosis factor alpha
TOT	Transovarial transmission
TPB	Tryptose phosphate broth (TPB)
UNIMAS	Universiti Malaysia Sarawak
UPR	University of Puerto Rico
UTMB	University of Texas Medical Branch
UTR	Untranslated region (UTRs)
V	Variable (Immunoglobulin region)
VEEV	Venezuelan equine encephalitis virus
VSV	Vesicular stomatitis virus
WBC	White blood cells
WHO	World Health Organization
WNV	West Nile virus
WRAIR	Walter Reed Army Institute of Research
WRCEVA	World Reference Collection of Emerging Viruses and Arboviruses
YFV	Yellow fever virus
ZIKV	Zika virus

CHAPTER 1: THE EMERGENCE OF INFECTIOUS VIRAL DISEASES: A GLOBAL PROSPECT WITH EMPHASIS ON MEDICALLY IMPORTANT ARBOVIRAL DISEASES

Emerging infectious diseases (EID) are described as infections that have recently appeared in a population, and are quickly increasing in frequency or geographic range (Morse 1995). For a disease to emerge, several factors are required, including the introduction of a pathogen and its spread into the human population, followed by its ability to be maintained in nature. Many pathogens require adaptation to emerge into a new environment, while for others adaptation is not necessary. Human behavior and ecology are two distinct factors that play a role in the emergence of diseases (Schrag and Wiener 1995; Hahn, Shaw et al. 2000; May, Gupta et al. 2001). For example, the geographical expansion of human populations has facilitated the appearance of emergent viruses, as well as the intensification of agriculture and the disturbance of habitats due to climate change and deforestation (Taylor, Latham et al. 2001; Jones, Patel et al. 2008).

It has been recognized that only a few infectious diseases are restricted to humans. The majority of emergent etiologic agents that affect humans are often maintained in enzootic cycles (Lloyd-Smith, George et al. 2009). In the past 70 years, emerging zoonoses made up most of the emerging infectious diseases identified in people and have caused economic damage exceeding hundreds of billions of U.S. dollars (Jones, Patel et al. 2008; Newcomb, Harrington et al. 2011; Karesh, Dobson et al. 2012). Zoonotic diseases account for billions of cases of illness and millions of deaths every year that constitute long-lasting health problems worldwide (Institute 2012).

The host range expansion of the zoonotic diseases requires multiples steps to establish transmission into the human population. Anthropogenic changes related to agriculture practices and deforestation are factors that bring humans in close contact with zoonotic reservoirs. Many wildlife species have been identified as reservoirs of important pathogens that can be transmitted to humans (Levins, Epstein et al. 1993; Morse 1994). For example, bats represent one major source of zoonotic viruses (Calisher, Childs et al. 2006), including Nipah virus (NiV), SARS coronavirus (SARS-CoV) and Ebola virus (EBOV) (Taylor, Latham et al. 2001; Woolhouse, Haydon et al. 2005).

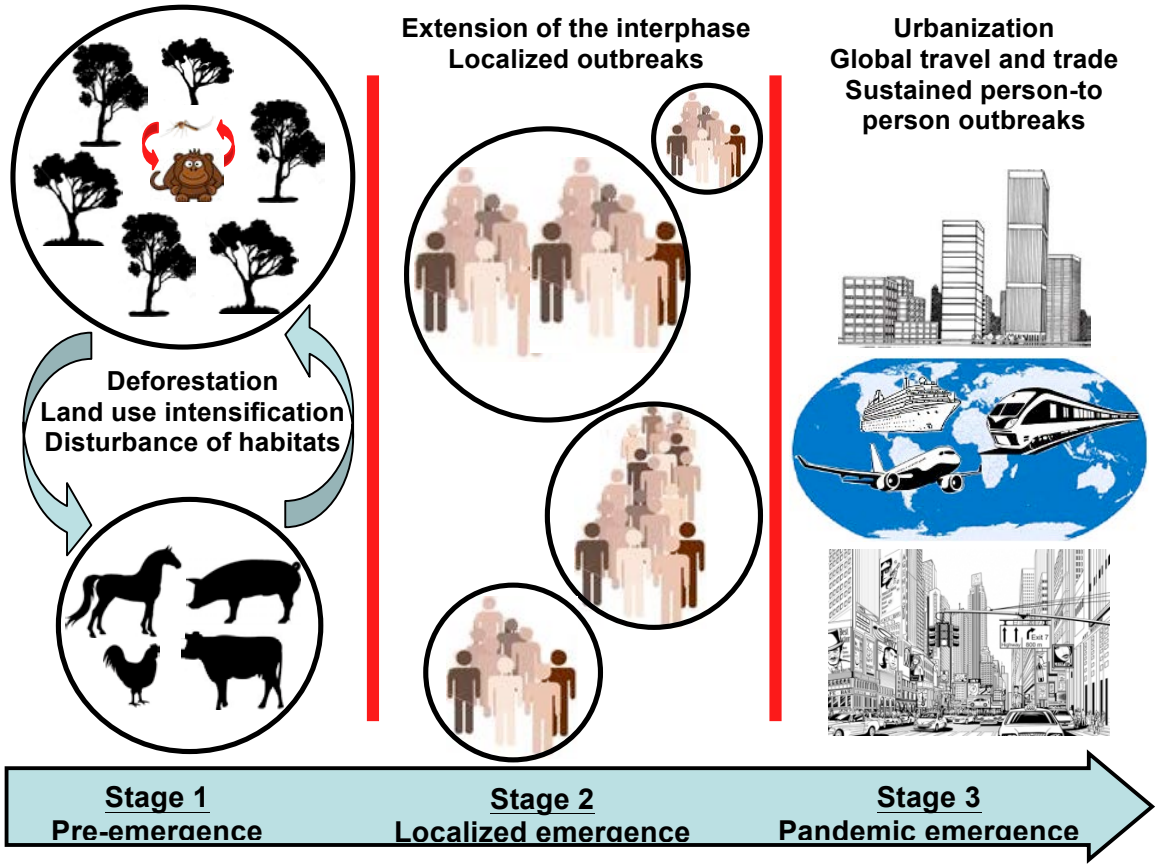

Figure 1.1. The emergence of infectious diseases into rural and urban areas. Distinct stages are implicated in the introduction of infectious diseases to human environments.

Many other zoonotic viruses are also transmitted to humans by hematophagous insects (mosquitoes, sandflies, black flies, biting midges, ticks) and are defined as arthropod-borne viruses (arboviruses) (Higgs and Beaty 2005). Most arboviruses affecting humans worldwide are classified into the families *Flaviviridae*, *Togaviridae* and *Bunyaviridae* (Blair, Adelman et al. 2000) and are transmitted mainly by *Aedes* and *Culex* mosquitoes (Kuno and Chang 2005). In recent years, the prevalence of vector-borne diseases has expanded considerably, due to intensification of human travel and transcontinental commerce. The number of cases has increased in endemic regions and also spread into new regions where the viruses never existed before (Gubler 2002; Weaver and Reisen 2010; Weaver 2013; Weaver 2014). Additionally, the development of mosquito resistance to insecticides has complicated even more the control and eventual elimination of diseases from specific areas (Saavedra-Rodriguez, Suarez et al. 2012; Bisset, Marin et al. 2013).

The Impact of Emerging Infectious Diseases

Since 1973, several reports of the World Health Organization (WHO) have identified at least 30 new infectious disease agents affecting humans; and for many of them no specific treatment is available to cure the infection (Satcher 1995; Murray 1996; WHO 1996; WHO 1998; Zaki, van Boheemen et al. 2012; Morrison 2014; PAHO 2015; WHO 2015; WHO 2015; WHO 2015). The impact of emerging infectious diseases (EIDs) not only as a public health threat, but also their economic burden, has both direct and indirect consequences. The total investment for the development of tools for early detection of pathogens, as well as, sustainable surveillance for potential pathogens emerging into a population, are factors that must be considered as a direct consequence of

EIDs economic impact. These costs are applied not only in diagnostic laboratory settings, but also directly in the field, hospitals or other point-of-care (POC) health care facilities. Examples of indirect costs accountable for the economic burden of EIDs are productivity losses from work absence, short-term disability and impairment of patient quality of life. Further steps following the introduction of EID should be considered such as, training of health care and other professionals dealing with the emerging pathogen, reducing the possibility of transmission to a larger population, and treatment responses, if available.

The World Economic Forum has listed the spread of EIDs as one of the top risk factors to cause potential economic loss to the world population (WEF 2015). Although the economic impact of EIDs is difficult to be accurately determined, several studies have been conducted to estimate their economic burden to society (Newcomb 2003; Zohrabian, Meltzer et al. 2004; Zohrabian, Hayes et al. 2006; Barber, Schleier et al. 2010). For example, during the emergence of severe acute respiratory syndrome (SARS) in 2003, in Asia, the virus rapidly spread in several countries in Asia, Europe and South and North America, in only a few months, affecting 8,098 people resulting in 774 deaths (CDC 2003). Its economic impact was estimated between 50 to 100 billion U.S. dollars (Newcomb 2003). The economic impact of the 2002 outbreak of West Nile virus (WNV) in Louisiana, which resulted in 24 deaths of the total 329 reported cases, was estimated to cost approximately to 20 million U.S. dollars. These costs included inpatient and outpatient visits, loss of work productivity, costs incurred by the public health department and mosquito control (Zohrabian, Meltzer et al. 2004; Zohrabian, Hayes et al. 2006). The spread of WNV in California and an outbreak in Sacramento County in 2005 resulted in 163 human cases, whose economic impact was estimated to be near 3 million U.S.

dollars, which included medical visits and treatment, job productivity loss and mosquito control (Barber, Schleier et al. 2010).

Additional studies have also attempted to anticipate the cost of potential outbreaks. In Australia, as one example of an isolated geographic area, the introduction of exotic diseases, as well as, pests and weeds could have a potential cost of over $1 billion Australian dollars (Murray, Skerratt et al. 2012). A study on the next influenza pandemic in the United States, estimated 89,000 to 207,000 deaths and an economic loss of 71.3 - 166.5 billion U.S. dollars. The cost was based on estimations for patient hospitalizations, outpatient visits and expenses for drug treatment and did not account for indirect costs interfering with commerce and community activities in affected areas (Meltzer, Cox et al. 1999). Overall, these examples highlight the impact EIDs can create for human populations and demonstrate the importance of controlling these diseases. One example would be the use of immunizations, when a vaccine is available.

The (re)-emergence of non-arboviral diseases

Several viruses transmitted by vertebrate hosts, including SARS and MERS coronaviruses, influenza, Ebola and HIV, have demonstrated the potential to (re)-emerge and cause a significant impact in the human population. SARS and MERS coronavirus (CoVs), members of the order *Nidovirales*, family *Coronaviridae*, are enveloped viruses containing a positive sense RNA genome of approximately 29,700 nucleotides long, infecting humans and a broad range of animals (Lai, Perlman et al. 2007). There are several hypotheses for the actual mechanism that CoVs use to jump from one species to another. Virus entry and innate immune response are the two most popular hypotheses playing a role in the process of CoV adaptation to a new host. However, the exact

mechanism employed for viral host switching is not completely understood (Muller, Raj et al. 2012; Raj, Mou et al. 2013). Direct zoonotic transmission from bats to human is not known to occur (Tang, Li et al. 2009). Therefore, the transmission involving other animals besides the known intermediate host (civets), for example carnivores or ungulates, although unusual, cannot be disregarded (Tang, Li et al. 2009; Graham and Baric 2010; Annan, Baldwin et al. 2013). Other factors, such as recombination should also be considered, which has been described in the emergence of feline infectious peritonitis viruses (FIPV) type 2 (Herrewegh, Smeenk et al. 1998) and has been speculated to play a role in the emergence of SARS-CoV (Hon, Lam et al. 2008; Graham and Baric 2010; Lau, Li et al. 2010; Yuan, Hon et al. 2010) and HCoV-OC43 genotypes (Lau, Lee et al. 2011).

Influenza virus, a member of the *Orthomyxoviridae* family, contains a segmented RNA genome, infecting mostly mammals and birds. Influenza types A and B are the two principal types of influenza virus. The type B of influenza infects only humans and is less common than influenza A (Hay, Gregory et al. 2001). The most pathogenic for humans is type A viruses, which are responsible for the majority of the observed severe disease. The classification of influenza viruses is based on the hemagglutinin (HA) and the neuraminidase (NA) proteins. Influenza A viruses contain 18 hemagglutinin and 11 neuraminidase subtypes (Brankston, Gitterman et al. 2007). H17N10 and H1N11 subtypes are reportedly found in bats; all others influenza A subtypes are found in birds. The source of all animal influenza A viruses is considered to be wild birds, which represent the primary reservoir to all influenza A viruses (Pascua and Choi 2014). Human strains of influenza virus can contain genomic material from different sources through

reassortment in swine, human and avian hosts. This reflects an adaptable property of the virus to change and re-infect new populations, as was recently documented in the human infections of H6N1 and H7N9 in Taiwan and China, respectively (Wei, Yang et al. 2013; Leung, To et al. 2015). The emergence of a new influenza pandemic is quite likely, because wild aquatic birds, the main reservoir of these viruses, cannot be eradicated. The rapid increase and concentration of human populations due to uncontrolled urbanization, as well as increased populations of swine and birds, have precipitated higher chances for virus exchange between species and for re-assortment of their genes (Lin and Wu 2015).

Ebola virus (EBOV), a member of the *Filoviridae* family, contains a single-stranded RNA genome and a lipid envelope and is responsible for a severe and highly contagious hemorrhagic fever in humans and other non-humans primates (Sanchez, Geisbert et al. 2007). EBOV was identified in 1976 in Central Africa and still a major threat to the world population. Fruit bats are considered the reservoir host and non-human primate hosts are important players in the maintenance of the virus in nature (Leroy, Epelboin et al. 2009; Olival, Islam et al. 2013). Some frugivorous and insectivorous bat species, recognized as reservoirs of the virus, may facilitate the transmission by contaminating fruits with their infected saliva, urine or feces that are later ingested by wildlife species (Leroy, Kumulungui et al. 2005; WHO 2014). Similarly, the unique behavior of chimpanzees make this species of NHP a very important player in the emergence of EBOV, as these animals are predators of other wildlife, hunting cooperatively and sharing meat among their social groups and carrying meat away from the place of predation. This particular behavior had previously associated with the occurrence of EBOV in Côte d'Ivoire, where several chimpanzees were affected. The

transmission of EBOV to humans usually occurs through direct contact with body fluids of infected animals, manipulation and ingestion of bushmeat, direct human contact during care or treatment of patients or preparation for burial (Bausch, Towner et al. 2007). In the recent Ebola outbreak in 2014, several factors have been associated with the exceptional expansion and emergence of EBOV in West Africa, including war, population growth, poor health infrastructure and poverty (Alexander, Sanderson et al. 2015). Another aspect influencing the spatial spread of diseases such as Ebola in West Africa is human migration across the landscape, generated by sociological and economic factors (Awumbila, Benneh et al. 2014). The rapid movement of infected people from their original infected village to other locations has led to human introduction of EBOV into major urban centers (Merler, Ajelli et al. 2015). The characteristic of transmission of EBOV is intensely affected by cultural and behavior factors that happen at the household, community and hospitals. Some countries, such as Liberia, consist of many cultural and ethnic groups, each representing its own language, dialect, religion and traditions (Johnston 2008). Specific practices and local rituals may have contributed to the spread on EBOV in those areas.

Human immunodeficiency virus (HIV), a virus that belongs to genus *Lentivirus*, family *Retroviridae,* has emerged into the human population as a consequence of zoonotic or cross species transmission, facilitated from different African primates infected with simian immunodeficiency virus (SIV) (Gao, Bailes et al. 1999). The various lineages of HIV that radiated for the first emergence events have been demonstrated to be more complex and volatile than first recognized and present the extraordinary properties of insidious disease initiation, persistence, recombination and escape from immune

system or pressure under drug exposure (Hahn, Shaw et al. 2000). It is not completely known how HIV first emerged into the human population, although recent studies suggest that occurred through the direct human contact with SIV-infected chimpanzees in Africa as early as the 1920s (Hahn, Shaw et al. 2000; Korber, Muldoon et al. 2000; Worobey, Gemmel et al. 2008). Current evidence also suggests that the introduction of HIV into the human population is derived from multiples events in different geographic areas, which have contributed to the emergence of distinct types and subtypes of HIV strains (Gao, Yue et al. 1992; Chen, Telfier et al. 1996; Gao, Bailes et al. 1999; Sharp, Bailes et al. 2000). Nevertheless, HIVs have accumulated an enormous sequence diversity, following its establishment of transmission in humans. This indicates that the virus evolution is occurring at a very rapid rate. Most of the genetic modification among HIV strains is due to properties of the viral replicative enzyme, reverse transcriptase, whose high mutation rate (Roberts, Bebenek et al. 1988; Abram, Ferris et al. 2010; Sanjuan, Nebot et al. 2010; Cuevas, Geller et al. 2015) is in the range of ca. 2.2 to 5.4 x 10^{-5} nucleotides changes per site per replication cycle (Mansky and Temin 1995; Gao, Chen et al. 2004). Consequently, these nucleotide substitutions play a critical role in contributing to the high genetic variability of HIV and highlight the remarkable plasticity and adaptive potential of its genome (Coffin 1992; Holland, De La Torre et al. 1992; Coffin 1995; Mansky and Temin 1995).

Zoonotic arboviruses and factors associated with their emergence

Arboviral diseases are caused by viruses that are maintained through transmission cycle between vertebrate hosts and blood-sucking arthropods such as mosquitoes, sandflies, midges and ticks. To complete the transmission cycle, it is essential that the

virus produce a sufficiently high level of viremia in the vertebrate host for the arthropod to become infected while taking a blood meal (Karabatsos 2001). There are at least 135 arboviruses that have been known to cause human disease. The infection can range from asymptomatic to severe and fatal disease. The clinical symptoms are generally categorized as systemic febrile illness, hemorrhagic fever and invasive neurological disease (Gubler and Vasilakis 2016). The vast majority of arboviruses are RNA viruses, belonging to several genera, including *Alphavirus*, *Flavivirus*, *Bunyavirus*, *Nairovirus*, *Phlebovirus*, *Orbivirus*, *Vesiculovirus* and *Thogotovirus*. Among DNA viruses, African swine fever virus (*Asfivirus* genus) represents the only DNA arbovirus (Calisher and Karabatsos 1988; King, Lefkowitz et al. 2011).

For arbovirus emergence to reach epidemic levels, it requires a recurrent interconnection of the susceptible vertebrate and invertebrate hosts within a permissive environment. Several factors involved in the emergence of arboviruses are related to distinct elements, such as the pathogen genetic selection, the susceptibility of the vector or host populations and anthropogenic influences that alter the environment configuration (land reclamation for agriculture, deforestation, etc) (Weaver and Reisen 2010).

In the last few decades, the total number of arboviral epidemics has significantly increased (Gubler and Vasilakis 2016). With the exception of few regions of the world not affected by arboviral epidemics, in most cases the emergence of arboviral diseases were caused by viruses previously considered to be controlled or recognized as harmless for the public health (Gubler and Vasilakis 2016). The expansion of the geographic areas where the mosquito vectors and viruses circulate have been associated with larger epidemics, such as dengue fever. Introduction of viruses into new geographic areas, e.g.

WNV in the United States, where naïve vertebrate and arthropod hosts were able to sustain infection also contributed to the occurrence of major outbreaks. In other cases, epidemics were associated with the regional dissemination of viruses initially contained into a restricted geographic area, e.g. Rift Valley fever, Ross River encephalitis, Japanese encephalitis and Venezuelan equine encephalitis. Other scenarios are represented by the potential of viruses to cause global public health problems due to regional advantages for viruses to maintain their transmission in specific regions, e.g. yellow fever in the urban areas of the tropics (Gubler and Vasilakis 2016).

Table 1.1. Examples of important arboviruses affecting humans.

Virus	Family	Vector	Vertebrate hosts	Geographic distribution	References
Chikungunya	*Togaviridae*	Mosquitoes: Aedes and Culex spp.	Primates, birds, cattle, and rodents	Africa, Asia, Europe, Americas, Oceania	37, 370, 410
Mayaro	*Togaviridae*	Mosquitoes: Haemagogus spp.	Primates, other mammals, birds	South and Central America	299, 384
Ross River	*Togaviridae*	Mosquitoes: Aedes and Culex spp.	Marsupials, other mammals, birds	Oceania and Asia	191, 301
O'nyong-nyong	*Togaviridae*	Mosquitoes: Anopheles spp.	?	Africa	300, 410
Sindbis	*Togaviridae*	Mosquitoes: Aedes, Culex, and Culiseta spp.	Birds	Europe, Africa, Oceania, Asia	96, 205
Barmah Forest	*Togaviridae*	Mosquitoes: Aedes and Culex spp.	Birds? Marsupials, Others?	Oceania	98, 275
Eastern equine encephalitis	*Togaviridae*	Mosquitoes: Culiseta, Aedes, Coquillettidia, and Culex spp.	Birds, horses, other mammals	Americas	49, 57, 442, 467
Western equine encephalitis	*Togaviridae*	Mosquitoes: Culex, Aedes, Ochlerotatus, and Coquillettidia spp.	Birds, horses, other mammals	Americas	57, 442, 467
Venezuelan equine encephalitis	*Togaviridae*	Mosquitoes: Culex, Ochlerotatus, Anopheles, Mansonia, Psorophora, Aedes spp. and others	Horses, Rodents, Other mammals, Birds	Americas	57, 442, 467
Dengue	*Flaviviridae*	Mosquitoes: Aedes spp	Primates	Asia, Americas, Africa, Europe, Oceania	48, 416
Yellow Fever	*Flaviviridae*	Mosquitoes: Aedes and Haemogogus spp.	Primates	South America, Africa	115, 461
West Nile	*Flaviviridae*	Mosquitoes: Culex spp	Birds, Horses, Other Mammals	Africa, Asia, Europe, Oceania, Americas	55, 217, 238, 345
Japanese encephalitis	*Flaviviridae*	Mosquitoes: Culex spp	Birds, Pigs	Asia, Oceania	99, 142, 168, 238
Murray Valley encephalitis	*Flaviviridae*	Mosquitoes: Culex spp	Birds	Oceania	52, 238, 355
Zika virus	*Flaviviridae*	Mosquitoes: Aedes spp	Primates	Africa, Asia, Oceania, Central and South America	42, 56, 148, 449
Rocio	*Flaviviridae*	Mosquitoes: Psorophora and Aedes spp	Birds	South America	252, 258, 362
St. Louis encephalitis	*Flaviviridae*	Mosquitoes: Culex spp	Birds, Bats, Other Mammals	Americas	54, 196, 316
Kyasanur Forest disease	*Flaviviridae*	Ticks: Hemaphysalis spp.	Primates, Rodents, Other Mammals	Asia	50, 159
Omsk hemorrhagic fever	*Flaviviridae*	Ticks: Dermacentor and Ixodes spp Mosquitoes: ?	Rodents, Volves, Other Mammals	Europe	53, 336
Tick-borne encephalitis	*Flaviviridae*	Ticks: Ixodes spp	Rodents, Goats, Sheep, Cows, Other Mammals, Birds?	Europe, Asia	27, 100
Sandfly fever	*Bunyaviridae*	Sandflies: *Phlebotomus* spp.	Birds? Mammals?	Europe, Asia, Africa	132, 398
Rift Valley fever	*Bunyaviridae*	Mosquitoes: Aedes, Ochlerotatus, Stegomyia, Anopheles, Culex, Neomelaniconion, Eretmapodites and others	Cows, Sheep, Camels, Goats and Other Mammals	Africa, Asia	277, 287, 376
La Crosse encephalitis	*Bunyaviridae*	Mosquitoes: Aedes spp	Rodents	North America	51, 146
Crimean-Congo hemorrhagic fever	*Bunyaviridae*	Ticks: *Hyalomma* spp	Cows, Sheep, Goats, Hares and Other Mammals	Europe, Asia, Africa	65, 358, 399, 447
Oropouche	*Bunyaviridae*	Midges: Culicoides sp	Primates? Sloths? Birds?	Central and South America	9, 265, 284, 411
Severe febrile thrombocytopenia syndrome	*Bunyaviridae*	Ticks: Haemaphysalis sp	?	Asia	372, 464, 466
Chandipura	Rhabdoviridae	Sandflies: *Phlebotomus* and *Sergentomyia* spp.	Hedgehogs, others?	Asia and Africa	103, 242, 254, 315, 383, 385
Bluetongue	*Reoviridae*	Midges: Culicoides spp	Sheep, Cows, Other Mammals	Africa, Asia, Europe, Oceania, Americas (all except Antarctica)	239, 285

WNV emergence in the Americas and Europe has been attributed mostly to commerce and dispersal of *Culex pipiens* complex mosquitoes, which are competent vectors of WNV transmission (Vinogradova 2000; Komar 2003; Fonseca, Keyghobadi et al. 2004). Other factors may include the introduction of house sparrows, climate change and the decline of SLEV (Weaver and Reisen 2010). The emergence of WNV was also associated with viral genetics, as the nucleotide sequence of the virus isolated in 1999 in the United States demonstrated a close relationship with a strain isolated in Israel in 1998, where a common mutation in the helicase gene was responsible for the high viremia and mortality in American crows (Brault, Huang et al. 2007).

Japanese encephalitis virus (JEV) is another arbovirus that has been associated with a significant public health impact following its emergence. JEV belongs to the genus *Flavivirus*, family *Flaviviridae* and forms a serocomplex including WNV. First described in Japan in the 1800s, JEV has expanded its geographic distribution in the Asian continent, Oceania and the Indian subcontinent (Burke and Leake 1988). There is discordance in how JEV evolution and distribution have occurred and which factors were responsible for the virus emergence and spread to different areas (Solomon, Ni et al. 2003). Increases in human population, and uncontrolled expansion of agricultural use, such as increases rice field acreage facilitated by irrigation and pig farming, were the most relevant factors associated with its rapid emergence (Erlanger, Weiss et al. 2009; van den Hurk, Ritchie et al. 2009). However, on the Indian subcontinent, birds may have played a major role in the emergence of JEV, because in these areas pig farming is limited (Boyle, Dickerman et al. 1983; Jamgaonkar, Yergolkar et al. 2003). In the

isolated Pacific islands of Guam and Saipan, air transport of mosquitoes has been correlated with the cause of JEV outbreaks (Mitchell, Savage et al. 1993).

In the case of yellow fever virus (YFV), the prototype virus of the genus *Flavivirus*, family *Flaviviridae,* phylogenetic studies demonstrated its African origin and dispersal to the New World by the slave trade during the 16th century (Monath 1988; Monath 2001; Bryant, Holmes et al. 2007). Numerous yellow fever epidemics occurred in port cities of North and South America in the early 17th century and were associated with the introduction of the *Ae. aegypti* vector. Non-human primates (NHPs) and canopy dwelling mosquitoes, such as *Hemagogus janthinomys* were responsible for the establishment of YFV sylvatic cycles in the Neotropics; despite immunization with the highly effective human vaccine 17D, both cycles continue at present days representing an unceasing risk of spillover to humans (Monath 2001; Bryant, Wang et al. 2003; Hanley, Monath et al. 2013). Despite exhaustive research to elucidate the factors attributed to YFV emergence, several questions remain unanswered. Severe and extended rainy seasons play a major role in YFV emergence in many geographic regions of the world, as exemplified in Nigeria and Brazil in 1987 and 2000, respectively. In Brazil, heavy rains and increasing temperatures precipitated the occurrence of epizootics and resulted in deaths among monkeys concurrent with human cases reported in several different states (Vasconcelos, Mota et al. 2000). Deforestation for land use in agriculture and cattle grazing are also contributing to the emergence of YFV, especially because these activities have brought humans in close contact with canopy dwelling mosquitoes inside or near the forest that are potentially infected with the virus (Najera, Oliva et al. 2013). In conclusion, it is essential to stress that the complex interactions between viruses, vector,

host, weather and environment remain only superficially understood in the emergence of YFV.

Rift valley fever virus (RVFV), a member of genus *Phlebovirus, family Bunyaviridae,* is responsible for sporadic epidemics in Africa. Sheep and cattle are severely affected by the disease, and humans are at risk of infection through spillover (Madani, Al-Mazrou et al. 2003). The factors associated with the emergence of this virus include warming caused by *El Nino* Southern Oscillation (ENSO) in the Pacific area and rainfall events, which cause the stimulation of hatching of a large numbers of mosquito eggs resistant to desiccation during drought seasons. This combination between rainy seasons and the presence of wild and domestic animals have guaranteed that mosquitoes have enough vertebrate hosts to feed on during warm humid weather conditions, making virus transmission successful and increasing the probability of it to emerge into vertebrate hosts including humans (Linthicum, Davies et al. 1985).

The emergence of epidemics caused by the Venezuelan equine encephalitis virus (VEEV), an alphavirus present only in the New World, is dependent on a combination of ecological factors and virus genetics that must interconnect in time and space (Weaver, Ferro et al. 2004; Anishchenko, Bowen et al. 2006). Since its first description as a disease during the 1930s in South America, it was reported periodically until 1973 (Walton and Grayson 1988). Subsequently, epidemics have occurred every 10–20 years, possibly due to the decrease of global herd immunity of equids generated either by lower vaccination efforts or absence of natural exposure to the virus (Anishchenko, Bowen et al. 2006; Weaver and Reisen 2010). Another factor associated with the emergence of VEEV is the enhancement of virus replication of enzootic strains in the amplification host (equids) by

the acquisition of specific mutations, such as the $E2_{213}T \rightarrow R$ (Brault, Powers et al. 2002; Anishchenko, Bowen et al. 2006). This allowed for the production of sufficient viremia to maintain the transmission cycle with the potential of spillover transmission to humans (Weaver and Reisen 2010). On the other hand, the emergence of VEEV is not exclusively dependent on viral genetics. Transportation of equids that carry and amplify the competent mutants to new areas where naïve equids and invertebrate vectors are abundant also is important to VEEV emergence (Weaver and Barrett 2004). Destruction of the tropical forest to expand ranching or other agriculture practices may also have contributed to increased risk of VEEV emergence (Weaver and Reisen 2010).

Another arbovirus that is increasing significantly and conquering new territories is chikungunya virus (CHIKV). It belongs to genus *Alphavirus*, family *Togaviridae*, and was historically found in the Old World (Jupp and McIntosh 1988). There are indications that the virus was originated in Africa, where it is believed that it is maintained in an enzootic transmission cycle between NHP and arboreal *Aedes* mosquitoes (Powers, Brault et al. 2000; Volk, Chen et al. 2010). Spillover transmission to the human population have occurred possibly multiple times resulting a continuous transmission cycle between humans and anthropophilic *Ae. aegypti* and *Ae. albopictus* competent vector mosquitoes (Diallo, Thonnon et al. 1999; Volk, Chen et al. 2010; Diallo, Sall et al. 2012). In 2004, CHIKV emergence was reported in the costal area of Kenya (Chretien, Anyamba et al. 2007) following a global expansion to different regions of Africa, Asia, several islands in the Indian Ocean (Hochedez, Jaureguiberry et al. 2006; Lanciotti, Kosoy et al. 2007; Taubitz, Cramer et al. 2007) and temperate areas in Europe (Rezza, Nicoletti et al. 2007; Grandadam, Caro et al. 2011). The contributing factor for the

emergence of CHIKV was presumably via travelers who became infected in endemic/epidemic areas and returned home contributing to the establishment of autochthonous transmission (Hochedez, Jaureguiberry et al. 2006; Lanciotti, Kosoy et al. 2007; Taubitz, Cramer et al. 2007).

Four genotypes of CHIKV have been identified since its discovery in 1952: East-Central-South African (ECSA), West African, Asian, and the Indian Ocean Lineage (IOL) (Powers, Brault et al. 2000; Volk, Chen et al. 2010). The different CHIKV lineages can exhibit distinct patterns of infectivity and transmissibility in the mosquito vectors (Arias-Goeta, Mousson et al. 2013; Vega-Rua, Zouache et al. 2013). The acquisition of specific mutations in the E1 (Tsetsarkin, Vanlandingham et al. 2007; Vazeille, Moutailler et al. 2007) and E2 (Tsetsarkin and Weaver 2011; Tsetsarkin, Chen et al. 2014) envelope glycoprotein of emerging IOL strains allowed virus adaptation and consequent increased transmission in the peridomestic mosquito *Ae. albopictus*. This adaptation may contribute to the spread and continuous transmission of CHIKV in tropical urban areas where *Ae. aegypti* is abundant and also to peridomestic and/or temperate habitats where *Ae. albopictus* is more adapted (Leisnham, LaDeau et al. 2014).

Despite the presence of both *Ae. aegypti* and *Ae. albopictus* mosquito vectors and reports of imported cases from the 2006-2009 period (Lanciotti, Kosoy et al. 2007) in the Americas, local transmission of CHIKV was only been reported recently. In 2013, an Asian lineage of CHIKV was introduced into the Caribbean island of Saint Martin and established the first mosquito-human cycle in the Americas (Leparc-Goffart, Nougairede et al. 2014). Subsequently, cases of autochthonous transmission of CHIKV were reported throughout the Caribbean and Central America, South America and Florida (Weaver and

Forrester 2015). In Brazil, two different CHIKV lineages were detected (Nunes, Faria et al. 2015). The Asian lineage reported in North Brazil possibly originated from travelers coming from the Caribbean, while the index case for the ECSA lineage reported in the northeast region (Bahia state) probably was introduced from a resident returning from Angola (Nunes, Faria et al. 2015).

Zika virus (ZIKV) is another arbovirus of the *Flaviviridae* family, genus *Flavivirus,* that is rapidly expanding its geographic distribution and has been recently introduced into areas not previously reported. The disease is characterized by a broad range of clinical symptoms, including fever, rash, headache, retro-orbital pain, myalgia, arthritis or arthralgia, conjunctivitis and vomiting, which are clinical signs similar to dengue disease and many other diseases of viral (e.g chikungunya and Mayaro fevers) and parasitic (e.g. scrub typhus and leptospirosis) aetiologies (Macnamara 1954; Olson, Ksiazek et al. 1981; Duffy, Chen et al. 2009; Foy, Kobylinski et al. 2011; Kutsuna, Kato et al. 2014). ZIKV was first isolated in 1947 from the blood of a sentinel rhesus monkey exposed in the canopy of Ziika Forest in Uganda during epidemiologic studies of yellow fever (Dick, Kitchen et al. 1952). Subsequent isolations of the virus were made from *Aedes africanus*, *Ae. luteocephalus* and *Ae. furcifer* (all tree-hole breeding mosquitoes implicated in the sylvan cycle of yellow fever virus) in Uganda, Senegal, Nigeria, Burkina Faso, Ivory Coast and the Central African Republic (Haddow, Schuh et al. 2012). These reports were interpreted as evidence that ZIKV is maintained in forested areas of tropical Africa in a cycle similar to that of sylvan yellow fever (i.e. arboreal mosquitoes and non-human primates). ZIKV was first isolated from humans in 1954 from a 10 year old Nigerian female (Macnamara 1954). The virus was isolated from mice

inoculated with the patient's serum sample; two other human cases were also confirmed from the same country. In 1969, ZIKV was isolated for the first time outside the African continent from *Ae. aegypti* mosquitoes collected in Malaya (Marchette, Garcia et al. 1969) and in 1977, the first human case was described in Indonesia (Olson, Ksiazek et al. 1981). The factors associated with the emergence of ZIKV are not understood. On the island of Yap, in Micronesia, where the first large outbreak was reported in 2007, ZIKV was speculated to have been introduced by either viremic travelers or infected mosquitoes originating from the Philippines, since travel exchange between Yap state and Philippines is very frequent.

In 2013 a major epidemic of ZIKV was reported in French Polynesia, where human subjects were presenting dengue-like symptoms and rash. Interestingly, few of the affected patients presented severe neurological complications and non-vector borne transmission (sexual and transfusion-associated cases) were also described (Cao-Lormeau, Roche et al. 2011; Musso, Roche et al. 2015). Although the total number of confirmed cases remains unknown, the number of patient consultations presenting symptoms of Zika fever was estimated to be about 28,000. A retrospective serosurvey, estimated the overall infection rate at 50-66% of the total population (Aubry, Teissier et al. 2015). The virus strain involved in French Polynesia outbreak was phylogenetically closely related to strains isolated in Yap and in Cambodia, suggesting that ZIKV could have been introduced from these regions (Cao-Lormeau, Roche et al. 2014; Musso, Nilles et al. 2014). In 2014, ZIKV cases were reported in New Caledonia in the South Pacific; unlike other Pacific regions where the virus source was unknown, in this outbreak the majority of the cases originated from individuals who have been in French Polynesia

(ProMEDmail 2014; Dupont-Rouzeyrol, O'Connor et al. 2015). In Easter Island, a local festivity that happens every year may have facilitated the introduction of ZIKV through people who came from several Pacific regions including French Polynesia (ProMEDmail 2014; Musso 2015). Following the introduction of imported cases from French Polynesia, other human infections were described and the presence of autochthonous cases of ZIKV was confirmed in the Cook Islands and on Easter Island in 2014 (ECDC 2014; ProMEDmail 2014; WHO 2015).

In 2015, ZIKV reached the Americas. The first country to report the virus was Brazil, where an outbreak of exanthematic disease was described and affected more than 6,000 people in Northeast region of that country (ECDC 2015; ProMEDmail 2015; Zanluca, de Melo et al. 2015). The state of Bahia was the first state to report autochthonous transmission of ZIKV; however, the virus easily spread across the country, where 14 states described autochthonous transmission (PAHO 2015; WHO 2015). Several factors may have played a role in the emergence of ZIKV in Brazil. The abundance of *Ae. aegypti* and *Ae. albopictus* vectors probably facilitated the virus emergence. There is speculation that ZIKV was introduced in Brazil through people attending in the 2014 World Cup, although many countries with reported cases of ZIKV did not participate in the competition (Salvador and Fujita 2015). Similarly athletes attending the World canoe championship, which took place in Rio de Janeiro, may also have been responsible for ZIKV's introduction, as many represented countries had major epidemics at the time (e.g. French Polynesia, New Caledonia, Cook Island and Chile). Concurrent phylogenetic analysis identified the Brazilian ZIKV as an Asian strain, suggesting that the virus may indeed have been entered Brazil through Asia or the South

Pacific (Musso 2015). Since ZIKV introduction in Brazil, autochthonous transmission has been reported in 31 countries/territories in the Americas (PAHO/WHO 2016).

Origin of dengue virus and dengue disease

The earliest evidence of dengue-like disease came from reports found in the Chinese medical encyclopedia dating back to AD 265-420 (further edited in AD 610 and AD 992) (Nobuchi 1979). The disease was linked to the presence of water-associated flying insects and thus named 'water poison'. Other reports of dengue-like disease were described in the West Indies in 1635 and in Panama in 1699 (Howe 1977; McSherry 1982). Following this period, numerous epidemics of disease resembling dengue were described in the continents of Asia, Africa and North America. Between 1779 and 1788, countries including Indonesia, Egypt, Spain and USA have reported dengue-like illness (Bylon 1780; Christie 1881; Hirsch 1883; Pepper 1941; Howe 1977) characterizing the wide geographic distribution of the disease.

The main urban dengue virus vector *Ae. aegypti* is believed to have had its origins in the African continent, suggesting that dengue virus was also originated in Africa (Gaunt, Sall et al. 2001). On the other hand, genetic studies showing the deep phylogenetic position of the Asian sylvatic strains the fact that the four serotypes are present in both humans and monkeys in Asia, strongly suggests that DENV had an Asian rather than an African origin (Wang, Ni et al. 2000).

In Asia, dengue viruses probably first emerged into the human population during deforestation practices for the establishment of agricultural settlements in areas adjacent to the jungle. The peridomestic *Ae. albopictus* mosquito was likely the bridge vector in the transmission of DENV in these areas (Gubler 2006). Consequently, human migration

and trade facilitated introduction and establishment of DENV transmission into more populated areas of tropical Asia, where the *Ae. albopictus* and other peridomestic *Stegomyia* mosquito species were abundant (Gubler 2006).

In Asia, as well as in the New World the introduction of the anthropophilic African mosquito *Ae. aegypti aegypti* was facilitated by the sea-borne and slave trade. Beginning in the 17th century, a wide distribution of *Ae. aegypti* was present throughout the tropics, starting in port cities and expanding inwards into the continent as part of the human urbanization expansion. As a result, a favorable environment was established for the transmission of DENV and major dengue epidemics have occurred, which rapidly became pandemics following World War II and continuing until now (Halstead 1992; Gubler 1997).

Following World War II, a new dengue-associated disease affecting predominantly children was described in endemic areas of Southeast Asia (Gubler 1998). An initial outbreak in Manila in 1953/1954, followed by a larger outbreak in Bangkok in 1958, provided the first clinical description of dengue hemorrhagic fever (DHF) (Hammon, Rudnick et al. 1960).

DENV (and Yellow Fever virus) epidemics in the Americas were restricted by a control campaign initiated in 1947 by the Pan American Health Organization (PAHO) aiming to eliminate *Ae. aegypti* from Central and South America. However, with the suspension of the control campaign in the 1970s, the region was reinfested with *Ae. aegypti* and the incidence of dengue started to rise again, reaching the pre-campaign levels by 1995. Since then the geographic distribution of dengue have increased not only in the Americas, but also in other regions of the world, from non-endemic to, in some

circumstances, hyperendemic levels (Gubler and Clark 1995; Gubler 2002; Shepard, Coudeville et al. 2011).

DENV transmission cycles

Dengue viruses are maintained in nature through two evolutionary and ecologicaly distinct transmission cycles: a sylvatic cycle, where the virus is transmitted among non-human primates by several arboreal *Aedes spp* mosquitoes, and the urban/human cycle, where virus transmission occurs between humans and mainly the domestic *Ae. aegypti* mosquito (Vasilakis, Cardosa et al. 2011).

The human transmission cycle is by far the most important cycle, considering its impact to public health and by the fact that it is occurring throughout the tropics. Although the *Ae. aegypti* mosquito is the major vector, the peridomestic *Ae. albopictus* and *Ae. polynesiensis* can play a role as secondary vectors of transmission (Gubler, Nalim et al. 1979; Gubler and Trent 1994). While currently *Ae. aegypti* is highly prevalent throughout sub-tropical and tropical areas, until the 15th century this vector was located exclusively in Africa (Lounibos 2002). The ability to adapt and survive in anthropogenic breeding sites, such as water storage containers in early agricultural settlements and later in ships, enabled this vector to take advantage of the slave trade and commerce to spread globally. Early reports support this notion as dengue fever was described as a disease of ports and coastal regions throughout the tropics and neotropics (Leichtenstern 1896). Linkage of *Ae. aegypti* mosquito with dengue disease was inferred from surveillance studies thorough several seaports of Southeast Asia and Indonesia (Theobald 1901; Stanton 1919). *Ae. aegypti* are anthropophilic and feed throughout the day and they lay their eggs in artificial containers usually found in domestic environments such as water

buckets, water storage cisternae and old tires. Human-to-mosquito DENV transmission depends on the magnitude of human viremia necessary to infect mosquitoes and their vector competence (Vazeille-Falcoz, Mousson et al. 1999; Bennett, Olson et al. 2002). Previous studies demonstrated that none or little transmission was achieved when the blood meal titer was below 10^3 viral RNA copies/ml and the level of transmission reached close to 100% when a dose was above 10^9 viral RNA copies/ml (Nguyen, Lee et al. 2013).

The capability of DENV to survive in dry climates during interepidemic periods has not completely elucidated; however there is evidence that the virus could be maintained through vertical transmission. The first evidence of arbovirus transovarial transmission (TOT) was demonstrated using phlebotomus flies and *Ae. triseriatus* in studies of vesicular stomatitis virus (VSV) and La Crosse encephalitis virus (LACV), respectively (Tesh, Chaniotis et al. 1972; Watts, Pantuwatana et al. 1973). The first evidence that DENV could be maintained in nature by TOT was with DENV type 2 (DENV-2) isolated from an *Ae. taylori* mosquito in Africa in 1980 (Roche, Cordellier et al. 1983). Subsequent studies in Senegal and Southeast Asia showed evidence of natural TOT of DENV-2 in *Ae. aegypti* (Khin and Than 1983). TOT was further demonstrated in India for DENV-3 and Trinidad Tobago for DENV-4 (Hull, Tikasingh et al. 1984; Joshi, Singhi et al. 1996) and later confirmed for the other serotypes (Thongrungkiat, Maneekan et al. 2011). TOT was also demonstrated for the four DENV serotypes in *Ae. albopictus* and for DENV-1 serotype in *Ae. aegypti* (Rosen et al., 1983).

Comparison of *Ae. aegypti* and *Ae. albopictus* populations from diverse geographic regions demonstrated substantial disparity in DENV susceptibility between

both vectors (Gubler and Rosen 1976; Gubler, Nalim et al. 1979). *Ae. aegypti* exhibited less susceptibility to DENV infection than *Ae. albopictus* (Gubler, Nalim et al. 1979; Jumali, Sunarto et al. 1979; Rosen, Roseboom et al. 1985; Gubler 1987). Moreover, the strain of the infecting virus could affect the susceptibility of the vector and the dynamic of DENV transmission (Gubler and Rosen 1977; Anderson and Rico-Hesse 2006; Hanley, Nelson et al. 2008). Considering the lower susceptibility of *Ae. aegypti*, the virus probably will need to achieve high viremia levels in the human host to be transmitted to mosquitoes during the blood meal, suggesting a natural selection of viruses that would potentially cause more severe dengue disease (Gubler 1987; Cologna, Armstrong et al. 2005).

Emergence of dengue virus

The emergence of DENV serotypes from a common sylvatic ancestor occurred thousand years ago, congruent with the establishment of early human settlements large enough to sustain transmission and was associated with vector changing from arboreal *Aedes* to peridomestic/domestic *Aedes spp.* and human reservoir hosts (Wang, Ni et al. 2000). Emergence of the serotypes occurred independently and repeatedly in allopatric regions prior to their expansion in sympatric regions, using similar non-human primate hosts (Vasilakis, Hanley et al. 2010; Vasilakis, Cardosa et al. 2011).

Phylogenetic studies demonstrated DENV was dispersed rapidly into new locations with the advent of air travel that enabled the movement of humans during the viremic phase of infection, resulting in the shift or extinction of local lineages (Rico-Hesse, Harrison et al. 1997; Carrington, Foster et al. 2005; Myat Thu, Lowry et al. 2005; Diaz, Black et al. 2006). Ecological factors are also involved in the emergence of DENV.

Deforestation is one of the major factors driving sylvatic DENV emergence. As people are exploring new resources deep into the forest, living in areas previously unexplored, the chances of sylvatic DENV emergence are also increasing (Patz, Daszak et al. 2004). In regions of Asia and Africa, where rapid and uncontrolled urbanization takes place, the risk of sylvatic dengue emergence is high.

Genetic diversity of dengue viruses

Genetic diversity and rapid evolution is a common thread among many RNA viruses. DENVs display a pronounced genetic diversity, as demonstrated by the presence of four distinct serotypes (Kuno, Chang et al. 1998). Phylogenetic analyses of the individual DENV serotypes also demonstrated the presence of multiples genetic subtypes (Rico-Hesse 1990; Wang, Ni et al. 2000; Twiddy, Farrar et al. 2002; Twiddy, Woelk et al. 2002; Holmes and Twiddy 2003; Twiddy, Holmes et al. 2003; Araujo, Nogueira et al. 2009; Vasilakis, Hanley et al. 2010; Villabona-Arenas and Zanotto 2011). DENV-1 is classified into five subtypes (I-V) (Rico-Hesse 1990; Chen and Vasilakis 2011); DENV-2 contains six subtypes (I-VI), with subtype III been further classified into sublineages IIIa and IIIb (Lewis, Chang et al. 1993; Salda, Parquet et al. 2005; Chen and Vasilakis 2011); DENV-3 contains four subtypes and DENV-4 is also classified into four subtypes (Lanciotti, Lewis et al. 1994; Lanciotti, Gubler et al. 1997). Multiples serotypes and subtypes are broadly dispersed and can cocirculate in different geographic areas (Rico-Hesse 1990; Lewis, Chang et al. 1993; Lanciotti, Lewis et al. 1994; Lanciotti, Gubler et al. 1997). This observed diversity is attributed mainly to the error-prone RNA-dependent RNA polymerase, generating on average one mutation per round of genome replication (Steinhauer, Domingo et al. 1992; Drake 1993).

Studies on selective pressures, measured as the ratio of nonsynonymous to synonymous amino acid substitutions, indicate that most DENV mutations are deleterious and subject to strong purifying selection (Holmes 2003). Conversely, the ability of DENV to adapt to changing niches or to engage new ecological niches undoubtedly could be generated from the genetic diversity. Consistently, the rate of substitution/site/year of DENV is calculated to be approximately $1x10^{-4}$, which is lower compared to numerous RNA viruses known to be transmitted directly between vertebrate hosts (Zanotto, Gould et al. 1996; Jenkins, Rambaut et al. 2002; Twiddy, Holmes et al. 2003; Bennett, Holmes et al. 2006; Dunham and Holmes 2007; Ramirez, Fajardo et al. 2010; Sall, Faye et al. 2010).

Recombination has also been suggested as a potential mechanism in the generation of DENV diversity. The concomitant geographic distribution of multiple subtypes of the same serogroup has been implicated to contribute with the occurrence of DENV recombination (Lorono-Pino, Cropp et al. 1999). In addition, the behavior of *Ae. aegypti*, which can feed multiple times on different hosts, also poses the potential for simultaneous exposure with more than one DENV strain and consequently increase the chances for recombination (Scott, Naksathit et al. 1997). On the other hand, recombination between viruses of distinct serotypes is unlikely, especially because the broad sequence divergence among the four DENV serotypes (Holmes and Burch 2000).

Recombination events have extensively been reported in DENV genomes (Holmes, Worobey et al. 1999; Worobey, Rambaut et al. 1999; Tolou, Couissinier-Paris et al. 2001; Uzcategui, Camacho et al. 2001; AbuBakar, Wong et al. 2002; Craig, Thu et al. 2003; Twiddy and Holmes 2003; Domingo, Palacios et al. 2006; Chen, Yu et al. 2008;

Perez-Ramirez, Diaz-Badillo et al. 2009); however, they have not been supported experimentally, but only by computational phylogenetic analyses, which requires caution when drawing conclusions about putative recombination among DENV genomes. Furthermore, demonstration of viable clonal recombinant flavivirus genomes using experimental approaches has been unsuccessful (Chuang and Chen 2009; Taucher, Berger et al. 2010; McGee, Tsetsarkin et al. 2011). The natural recombination leading to the transmission of recombinant genomes should be confirmed by demonstration of the recombinant crossover at molecular level, detection of the recombination multiple times in clonal populations of viable virus and demonstration of sequence preservation during post-recombination evolution. One of the most persuasive events of DENV recombination was described in the Pacific region of New Caledonia where multiple DENV-1 isolates containing both parental and recombinant viruses were detected in the same patient (Aaskov, Buzacott et al. 2007). The data was verified by phylogenetic analyses with further genetic confirmation of identical crossover break points (Aaskov, Buzacott et al. 2007).

Genetic diversity can also be introduced in a population through the migration process. The increase and concentration of the vector and human population, combined with increase in transportation have provided more opportunities for DENV dissemination and diversification in highly populated areas. Historically, dissemination of mosquito vector in Southeast Asia in the 1950's and extensive movement of population, mainly battle troops in the World War II are examples that support the importance of migration in building the DENV antigenic diversity (Rosen 1977; Gubler 1997).

The re-introduction of DENV in the Americas was greatly accelerated by the termination of eradication programs implemented in the 1970s, which consisted in the extinction of *Ae. aegypti*, the primary DENV vector. As a consequence, the mosquito was re-introduced and re-colonized in several regions of Central and South America. Additionally, several Latin American countries progressed from non-endemic or hypo-endemic to hyperendemic in the 1980s and 1990s (Lorono-Pino, Cropp et al. 1999). Hence, the substantial augmentation of genetic diversity could be the result of serotypes and genotypes introduction into extended geographical areas where strains genetically distant are already endemics (Chen and Vasilakis 2011).

Antigenic relationship of dengue viruses

Historically, flaviviruses were classified into serocomplexes based on serologic relationships, such as the virus neutralization profile (Calisher, Karabatsos et al. 1989). The serological cross-reactivity is determined by the presence of common epitopes in the virion surface (Trent 1977). The envelope (E) glycoprotein is considered the major target of flavivirus neutralizing antibodies (Pierson, Fremont et al. 2008). The classification of DENV into distinct serotypes was based on observations that following primary DENV infection, the monotypic immune response generates a full protection against homologous viruses, but partial and transient protection, lasting for only a few months, against heterologous DENV strains (Sabin 1952). As a result, a single person can potentially be infected with all four DENV serotypes during her lifetime (Rothman 2011).

Antigenic differences among viruses are determined by amino acid substitutions that can promote changes in the protein structure and consequently altering the

recognition and binding of antibodies. Among the DENV serocomplex, the amino acid sequence of E protein can diverge by up to 37%, but contains conserved regions that can induce the production of cross-neutralizing antibodies (Heinz and Stiasny 2012). The modifications on the protein structure that lead to an antigenic effect can be the result from a single to multiples amino acid substitutions and sometimes amino acid changes have no antigenic effect (Koel, Burke et al. 2013; VanBlargan, Mukherjee et al. 2013). Therefore, it is difficult to predict differences in antigenicity only from the genetic sequence, so the characterization must include an antibody neutralization assay to determine the antigenic relationship among viruses (Calisher, Karabatsos et al. 1989).

To determine the antigenic relationships among the DENV, it is common to represent their neutralization profile against a panel of several different sera know to react with specific DENV types (Vasilakis, Durbin et al. 2008). It has been demonstrated that sera obtained from humans during a primary infection or immunized with DENV exhibit strong homotypic neutralization against different urban and sylvatic DENV, where the heterotypic neutralization is absent or last for a short period of time (Vasilakis, Durbin et al. 2008). However, many times these analyses are difficult to interpret due the intrinsic variability among samples derived from different hosts or infection histories (Thomas, Nisalak et al. 2009; van Panhuis, Gibbons et al. 2010). More recently, the antigenic relationships of DENV have been studied using antigenic cartography to reduce some measurements errors of neutralization against multiple serotypes (Katzelnick, Fonville et al. 2015). The analyses of a panel of human and non-human primate sera derived from experimental infection, as well vaccination and natural infection demonstrated that the majority of DENV isolates were clustered into each DENV type

classification. However, a number of viruses were located more adjacent to another DENV type than its own type and the distance within and between types was similar. The neutralization profile of antisera demonstrated similar trend, with groups close to the homologous virus type, but also close to a heterologous DENV (Katzelnick, Fonville et al. 2015).

Requirements for dengue emergence?

The four currently known DENV serotypes are considered to be originated from independent evolutionary events that most likely occurred repetitively in Asia, resulting in the emergence of endemic DENV derived from sylvatic ancestors (Vasilakis and Weaver 2008). Vector switching from arboreal primatophilic mosquito species to peridomestic mosquito vectors (*Ae. aegypti* and *Ae. albopictus*) is also attributed to have facilitated the emergence and adaptation of sylvatic strains into the urban transmission cycle (Wang, Ni et al. 2000). The expansion of non-human primates and human populations in different geographic areas allowed the sustained transmission of DENV into the major tropical regions of the world.

The *Ae. albopictus* was most likely the primary human mosquito vector responsible for DENV transmission. With the advance of navigation and consequent expansion of commercial trade routes, *Ae. aegypti* also start to colonize the tropical areas, especially in port cities where dengue became endemic. To understand if adaptation of sylvatic DENV was necessary to sustain transmission in these peridomestic vectors and establish an urban cycle, Moncayo et al. evaluated vector competence using endemic and sylvatic DENV-2 strains to infect *Ae. albopictus* and *Ae. aegypti* mosquitoes originated from southeast Asia and Americas (Moncayo, Fernandez et al. 2004). The study showed

a higher susceptibility of *Ae. albopictus* to endemic DENV-2 strains than *Ae. aegypti* independently of mosquito location. However, when data from both mosquito groups were combined, although endemic DENV-2 have higher rates of infection, the dissemination of sylvatic and endemic strains from the midgut were similar between the two mosquito species, suggesting no need for adaptation of sylvatic DENV to sustain transmission in urban transmission cycle.

The possibility of sylvatic strains to enter the human transmission cycle was also recently evaluated by both *in vitro* and *in vivo* human models of DENV replication. The purpose of those studies was to verify if any adaptation is required to sylvatic DENV strains been established in a new transmission cycle. DENV-2 infection of human monocyte-derived dendritic cells (moDCs) and severe combined immunodeficient (SCID) mice xenografted with human hepatoma (Huh-7) cells demonstrated no significant differences in replication between sylvatic and human strains (Vasilakis, Shell et al. 2007). Interestingly, sylvatic DENV-2 replication in moDCs was comparable with human DENV-2 strains, suggesting they can promptly infect human hosts (Vasilakis, Shell et al. 2007). Other study using cell lines representing human (Huh-7), monkey (Vero) and mosquito (C6/36) hosts demonstrated that the human strains only have higher level of viral replication in the human cell, but virus titer were similar in the monkey and mosquito cell lines (Vasilakis, Fokam et al. 2008). Collectively, these studies demonstrate the ability of sylvatic DENV strains to replicate in a range of host cells, suggesting that their emergence in the human population is not dependent on adaptation to new hosts, but most dependent on the opportunity of the sylvatic virus to infect a wide range of hosts and eventually emerge into a human transmission cycle.

Conclusions

The majority of viruses with potential to produce important epidemics are zoonotic, which means that they are originated in an animal hosts and are driven by several emergence forces, including changes in ecological and social behaviors, supporting the possibility to spill over into the human population. To clearly comprehend and anticipate the occurrence of sylvatic DENV emergence is fundamental to clarify the ecological and epidemiological aspects related to this virus cycle. There is enough evidence to support the existence of endemic serotypes as a result of independent events through cross-species transmission of sylvatic DENV. However, there is clear indication that sylvatic DENV come into close contact with humans in Asia and Africa, and possibly in other parts of the world, originating sporadic severe dengue disease that can spillover in the urban environment.

The sylvatic cycle of DENV has not being intensively explored and not considerable attention is given to the consequences involved in viruses coming from unexplored habitats. Additionally, different of what was proposed in the past, recent studies indicated that the emergence of sylvatic DENV represent a real threat to people considering the inexistence of an adaptation barrier to sylvatic viruses emerge into the human population. Moreover, the diversity of DENV strains and the emergence of new isolates have important consequences in the development of therapeutics, including vaccines currently in the developmental and clinical trial phases.

CHAPTER 2: Discovery and Characterization of a Novel Dengue Virus Serotype

INTRODUCTION

The four extant DENV serotypes that represent both sylvatic and urban transmission cycles emerged from sylvatic strains in the forests of Southeast Asia. The emergence of the ancestral viruses is believed to have occurred repeatedly over time congruent with the establishment of human settlements large enough to support a self-sustained transmission cycle several thousand years ago. The persistence of these ancestral strains, which are still extant in both Southeast Asia and West Africa, enables us to study those initial emergence events. However, continued circulation of sylvatic strains also poses a risk for the contemporary emergence of sylvatic strains into the human population.

Previous experimental work in the Vasilakis laboratory demonstrated that: (1) emergence of endemic DENV strains from ancestral sylvatic strains may not have required adaptation to replicate more efficiently in human reservoir hosts (Vasilakis, Shell, et al., 2007) or mosquitoes (unpublished data); (2) endemic and sylvatic DENV-2 share similar rates of evolutionary change and patterns of natural evolution (Vasilakis, Holmes, et al., 2007); (3) unrecognized outbreaks of sylvatic DENV-2 are taking place in urban settings (Vasilakis, Tesh, & Weaver, 2008); (4) there is robust homotypic cross-immunity between human sera (from natural DENV infections as well as vaccinees) and sylvatic DENV (Durbin et al., 2013; Vasilakis, Durbin, et al., 2008); (5) sylvatic and endemic DENV-2 and DENV-4 do not share significant differences in their progeny

output in vertebrate or invertebrate hosts (Durbin et al., 2013; Vasilakis, Fokam, et al., 2008); and (6) slower than expected rates of mutation accumulation for both endemic and sylvatic DENV occur in mosquitoes (Vasilakis et al., 2009). Collectively, these lines of evidence imply that the sylvatic cycles in Asia and West Africa will remain a source of re-emergence. Although the currently licensed DENV vaccine and others under development may facilitate the eradication of endemic DENV strains (because humans are the only reservoir hosts) sometime in the not so distant future, the sylvatic strains are not amenable to control and will probably remain a source of re-emergence. Nonetheless, human herd immunity from repeated exposure to endemic DENV strains and immunity generated by some vaccines is capable of sylvatic strain neutralization, indicating that sustained vaccination may be able to prevent future re-emergence into the human transmission cycle.

Serendipitous investigations of febrile illness in Senegal and Southeast Asia suggest that sylvatic DENV infection can cause dengue (DEN) disease. Almost all of these cases were concurrent with amplification of sylvatic DENV in arboreal *Aedes* mosquitoes [reviewed in (Vasilakis, Cardosa, Hanley, Holmes, & Weaver, 2011; Vasilakis, Hanley, & Weaver, 2010; Vasilakis & Weaver, 2008)]. Importantly, these cases demonstrated that illness due to sylvatic DENV infection is indistinguishable from classic dengue fever (DF) infection with the ecologically and genetically distinct DENVs from the human transmission cycle. However in 2008, two events were linked to sylvatic dengue virus activity in humans in West Africa (Franco et al., 2011) and Southeast Asia (Cardosa et al., 2009) demonstrated that sylvatic DENV infection can also lead to severe DEN disease. The implications from both cases are significant because: (i) the virus

responsible for the human infection in Southeast Asia was maintained in nature without detection for nearly 4 decades; (ii) they represent the first documented human cases that sylvatic DENV infections can cause severe DEN disease; (iii) both confirm our previous evidence from West Africa that re-introduction of sylvatic DENV into the human transmission cycle is possible (Vasilakis, Tesh, et al., 2008).

The aim of this study is to describe and characterize the discovery of a novel new dengue virus isolated from a febrile patient in the Malaysian state of Sarawak, which presumably represents the prototype virus of a new dengue serotype.

MATERIALS AND METHODS

Viruses

DENV isolates included in this study were the following: DENV-1, Hawaii; DENV-2, NGC; DENV-3, H87; DENV-4, H241, LF32 and 1120; DENV-5, DKE121. Low passage viruses were propagated in C6/36 cultures to obtain high titer stocks. Cell supernatants were clarified from cellular debris by low-spin centrifugation (630 x g, 10 min at 4°C), stabilized with the addition of 1X SPG (2.18 M Sucrose, 0.038M KH_2PO_4, 0.072M K_2HPO_4 and 0.054M L-glutamate), aliquoted and stored at -80°C. Viral stocks were quantified by focus-forming assay (FFA) as previously described (Vasilakis, Shell, et al., 2007).

Next Generation Sequencing

Confluent monolayers of C6/36 cells (T25 flask) were infected with DKE-121 and harvested 7 days later when CPE was observed. Viral RNA was prepared and processed for sequencing as described previously (Vasilakis, Forrester, et al., 2013). The

de novo assembly program ABySS (Simpson et al., 2009) was used to assemble the reads into contigs, using several different sets of reads, and k values from 20 to 40. A nearly full-length contig was obtained from 150,000 reads and a k value of 37. Reads were mapped back to the contig using bowtie2 (Langmead & Salzberg, 2012), and visualized with the Integrated Genomics Viewer (Robinson et al., 2011) to verify that the assembled contig was correct. About 3.5% of the reads in the sample mapped to the viral contig, resulting in about 2.1 million reads mapped out of about 60 million total.

Phylogenetic Analysis

The novel DENV genome was manually aligned to a subsample of representative full genome sequences of each genotype along with all available full genome sequences of sylvatic origin obtained from GenBank, using Se-AL (version 2.0a11 Carbon, http://tree.bio.ed.ac.uk/software/seal). Sequences were examined for evidence of recombination using the Bootscan, Chimaera, GENECONV, MaxChi, RDP, and SisScan methods with default parameters, implemented in the RDP3 software package (Martin et al., 2010), in which potential recombinant sequences are identified when three or more methods within RDP3 were in agreement with $P<0.001$. No evidence of recombination was shown within the dataset. The phylogenetic tree was inferred from nucleotide sequences under the maximum likelihood (ML) method available in PhyML (Guindon & Gascuel, 2003), using the GTR+G4 model of nucleotide substitution with SPR branch-swapping and 1000 bootstrap replications, as determined by ModelTest (Posada & Crandall, 1998). An additional phylogenetic tree (not shown) was constructed from amino acid sequences using the JTT+G4 model of amino acid substitution with SPR branch-swapping and 1000 bootstrap replications, as determined by ModelGenerator

(Keane, Creevey, Pentony, Naughton, & McLnerney, 2006). P-uncorrected nucleotide and amino acid differences were calculated using Geneious (v8.1.7).

Generation of Mouse Hyperimmune sera

Specific hyperimmune mouse ascitic fluids were prepared by four intraperitoneal injections to CD1 mice, given at weekly intervals, with 10% suspensions of homogenized infected mouse brain in PBS mixed with Freund's adjuvant. Sarcoma 180 cells were given intraperitoneally after the final immunization to induce ascites formation.

Plaque Reduction Neutralization Test (PRNT)

PRNTs were performed in 24-well plates, as previously described (Durbin et al., 2013). Briefly, constant virus amount (1,500 FFU/mL) was mixed with an equal volume of 2-fold dilutions (1:10-1:2,560) of human serum or mouse hyperimmune ascitic fluid (MIAF). Human serum samples were obtained from patients with known monotypic DENV-1 to -4 infection (Iquitos, Peru) or from a panel of human sera containing naïve (flavivirus negative samples), Japanese encephalitis virus (JEV)-exposed, monotypic DENV-1 to -4 or polytypic DENV infection (Armed Forces Research Institute of Medical Sciences – AFRIMS, Thailand). MIAF were raised against homologous DENV-1 to -5 antigens. The serum-virus mixture was incubated 1hr at 37°C. Subsequently, 100 µL of the mixture was transferred into 24-well plates containing Vero cells at circa 70% confluence and incubated 1hr at 37°C. A 1.0 mL volume of 0.8% methyl cellulose in OPTIMEM-I (GIBCO) overlay was placed in each well and the plates were incubated at 37°C for 4-5 days depending on the virus phenotype. The cells were then fixed with acetone and methanol (1:1) solution and immune-stained as previously described (Vasilakis, Durbin, et al., 2008). The PRNT titers were scored as reciprocal of the highest

dilution of serum that inhibited 80% of foci ($PRNT_{80}$) according the NIAID Laboratory of Infectious Diseases (LID) Plaque Reduction Web Tool available at exon.niaid.nih.gov/plaquereduction/index.html.

Antigenic Cartography

The antigenic cartography maps were generated as described previously (Katzelnick et al., 2015). Briefly, each neutralization titer N_{ij} was transformed into a table antigenic distance D_{ij} between virus *i* and antiserum *j* by calculating the difference between the titer for the virus best neutralized by each antiserum *j*, defined as b*j*, and the measured titer for each virus N_{ij} against that antiserum: $D_{ij}=\log_2(b_j)-\log2(N_{ij})$. To find the map distances, represented by the Euclidean distance d_{ij} between each virus *i* and antiserum *j*, the differences between the map and table distances were minimized, as defined by the error function $E=\sum_{ij} e(D_{ij},d_{ij})$. The error of a serum-virus pair was defined as $e(D_{ij},d_{ij})=(D_{ij}-d_{ij})^2$ when the neutralization titer was numeric (meaning that neutralization titer was within the limit of detection of the neutralization assay). The error was defined as $e(D_{ij},d_{ij})=(D_{ij}-1-d_{ij})^2$ $(1/1+e-10(D_{ij}-1-d_{ij}))$ for threshold titers (defined as titers below the limit of detection, <1:10), so that the titer contributed to the stress only if the map distance was less than the minimum specified target distance ($d_{ij} < D_{ij}-1$). Further description of the antigenic cartography technique has been published previously (Fouchier & Smith, 2010). To identify the antigenic map for which the distances between viruses and antisera most closely matched the table distances, viruses and antisera were assigned random starting coordinates and the error function was minimized using the conjugate gradient optimization method. In order to increase the likelihood of finding a good minimum, 5,000 independent optimizations were performed. The observed

minimum error map is demonstrated on a grid matrix in which each square side corresponds to a two-fold antiserum dilution, or one antigenic unit (AU), in any direction, on the antigenic map. Thus, two grid square sides are a four-fold antiserum dilution, three grid square sides are an eight-fold antiserum dilution, and so on.

RESULTS AND DISCUSSION

Case Description

On 21 May 21, 2007, a 36 year old male Bidayuh farmer residing in the state of Sarawak, Malaysian Borneo, first became ill exhibiting symptoms of high fever, chills and rigor. On day 3 post onset of symptoms, when illness persisted with headache, retro-orbital pain, myalgia, arthralgia and 2 episodes of spontaneous but self-limiting gum bleeding, the patient presented into the nearby community clinic. When full blood count showed leukopenia and thrombocytopenia, he was referred to Sarawak General Hospital located in the state capital of Kuching, which was 45 km away. On examination, he looked flushed, mildly dehydrated, with a blood pressure (BP) of 120/90 mm Hg, pulse 86/min, low platelet count and elevated liver enzymes, body temperature 37.8°C rising to 38.2°C the next day before defervescence by the fifth day after onset (Table 2.1). He presented with mild non-tender hepatomegaly, 1 cm below costal margin, and no other abnormalities. He was diagnosed with grade II dengue hemorrhagic fever based on WHO dengue diagnosis guidelines, was treated and discharged after remaining well for 36 hrs after defervescence. Although at the time no attempt was made to screen by PCR, the sample was biobanked for further investigation at the Institute of Health and Community Medicine (IHCM) at the Universiti Malaysia Sarawak (UNIMAS). Further investigation

by the public health authorities showed that other villagers also fell ill with similar symptoms and vector control had carried out fogging activities in the area.

Table 2.1: Results of the laboratory investigation

	Day of illness					
	05/24/2007 (OPD_RCBM)	05/24/2007 (A&E Dept)	05/25/2007 (Ward)	05/25/2007 (Ward)	05/26/2007 (Ward)	05/26/2007 (Ward)
Hemoglobin (g/dL)	18.6	11.8	16.5	15.3	15.8	16.2
Total white cells (per μL)	4800	3900	5100	5400	13500	10300
Platelets (per μL)	51000	59000	40000	21000	32000	61000
Hematocrit (%)	-	36.3	49.0	46.3	46.5	50.7
Lymphocytes (%)	-	19.7	-	-	-	-
Neutrophils (%)	-	75.1	-	-	-	-
Prothrombin (sec)	-	-	13.4	-	-	-
PTT (sec)	-	-	59.1	-	-	-
Sodium (mmol/L)	-	136	140	-	-	-
Potassium (mmol/L)	-	4.3	4	-	-	-
Chloride (mmol/L)	-	103	100	-	-	-
Urea (mmol/L)	-	-	4.8	-	-	-
Creatinine (umol/L)	-	-	131	-	-	-
Total Bilirubin (μmol/L)	-	-	6	-	-	-
AST (U/L)	-	-	317	-	-	-
ALT (U/L)	-	-	74	-	-	-
Total protein (g/L)	-	-	83	-	-	-
Albumin (g/L)	-	-	46	-	-	-
Alkaline phosphatase (U/L)	-	-	69	-	-	-
DENV PCR	-	-	-	-	-	-
Virus isolation	-	-	-	-	-	-

Abbreviations: PTT - Partial Thromboplastin Time; AST - Aspartate aminotransferase; ALT - Alanine aminotransferase; DENV – dengue virus; PCR – polymerase chain reaction

Genomic Sequence and Phylogenetic Analysis of the Novel Dengue Virus

In 2009, scientists at IHCM started a retrospective characterization of biobanked samples. Based on serologic diagnostic assays used at that time and the sequencing and BLASTing of a poorly amplified PCR product (Johnson, Russell, & Lanciotti, 2005), the patient's sample was misclassified as DENV-4. Further attempts to obtain the full genomic sequence of this isolate were unsuccessful. The sample, designated as DKE-121, was then send for further characterization at the Vasilakis laboratory at the University of Texas Medical Branch. Upon receipt, the virus was amplified in C6/36 cells to generate adequate virus stocks. The full genomic DENV sequence of DKE-21 was

later obtained by *de novo* next generation sequencing (NGS) using the laboratory's developed protocols (Vasilakis, Forrester, et al., 2013; Vasilakis, Widen, et al., 2013). The genome is comprised of 10,677 nucleotides (nt) of single stranded RNA of positive polarity. A single open reading frame (ORF) of 10,164 nucleotides is flanked by untranslated regions (UTRs) at both 5' and 3' ends. The 5'- UTR is 101 nt long and the 412 nt 3'-UTR lacks a polyadenylation site. The ORF encodes three structural (capsid (C), premembrane/membrane (prM/M) and envelope (E)) and seven nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins. The sequences of both the 5' and 3' UTR are highly conserved and sequence motifs and secondary structural elements [such as the RCS2, CS2 and CS1, as well as the pentanucleotide sequence (CPS) within the 3' UTR stem-loop (SL)] within these regions are conserved among all flaviviruses (data not shown) [reviewed in (Rossi et al., 2012; Vasilakis, Fokam, et al., 2008)]. Comparison of both nt and amino acid (aa) sequences from representative taxa from each genotype of the known four dengue serotypes suggested significant divergence (Table 2.2). Genome-wide divergence is observed to greater between DKE-121 and serotypes 1-3 than between DKE-121 and DENV-4, suggesting a closer genetic and possibly antigenic relationship. Similar observations were made with comparisons at the gene level (Figure 2.1).

Table 2.2: Percent (%) nucleotide and amino acid identity genome-wide between DKE-121 and viruses from representative taxa of dengue serotypes 1-4.

	nucleotide	amino acid
DENV-1	65.5 - 66.0	68.5 - 69.1
DENV-2	65.6 - 66.2	68.7 - 69.2
DENV-3	66.0 - 66.4	69.3 - 69.5
DENV-4	74.9 - 75.2	85.0 - 85.4

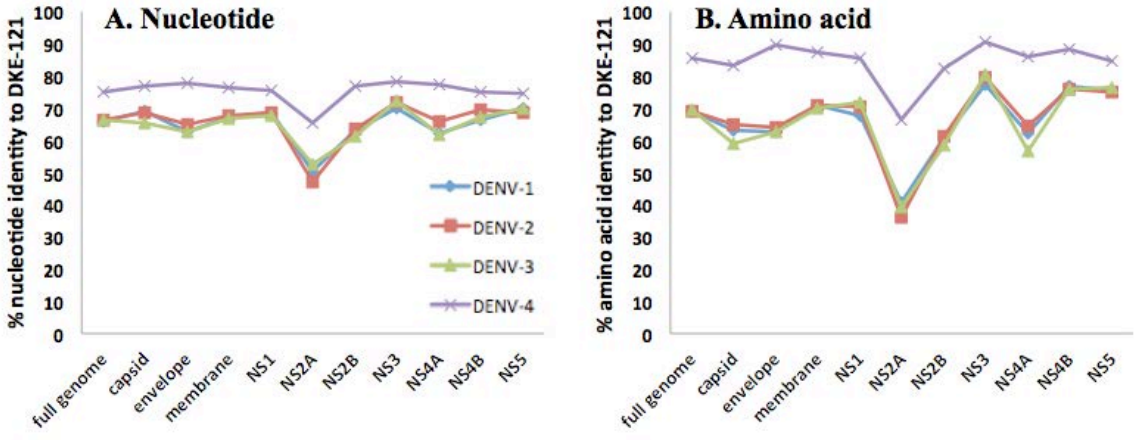

Figure 2.1: Gene-specific percent nucleotide and amino acid identity between DKE-121 and viruses from representative taxa of dengue serotypes 1-4.

The next step in the characterization of this isolate was to determine its phylogenetic relationship to other DENV. The open reading frames (ORF) of 64 representative strains from both sylvatic and human transmission cycles were manually aligned using Se-AL and their phylogenetic relationships were inferred by maximum likelihood (ML) as described in the materials and methods section above. While phylogenetic analysis of the virus isolate (DKE-121) strongly suggests its sylvatic origin (black arrow, Fig. 2.2), it is substantially divergent from its closest relative, sylvatic DENV-4. Importantly the branch length of the divergent DKE-121 is deeper than the branch length of DENV-2 emerging from its ancestral progenitors but shallower than the branch lengths of the sister serotypes DENV-1 and DENV-3, suggesting the unique origin of this virus and implying the emergence and detection of a new dengue serotype.

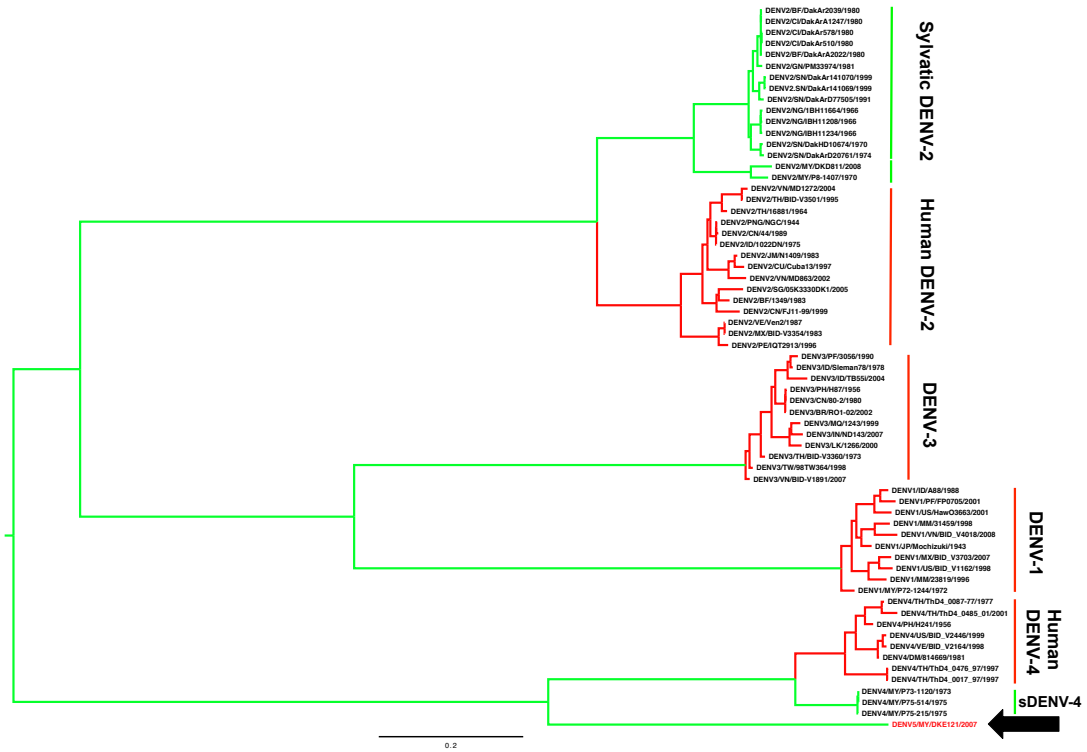

Figure 2.2: Evolutionary relationships of DKE-121. Phylogeny derived from complete ORF sequences. Green branches indicate predicted ancestral sylvatic lineages and red branches indicate human lineages.

This human infection (sample DKE-121), along with the sylvatic DENV-2 infection described above (Cardosa et al., 2009) and the newly documented and highly divergent lineage of sylvatic DENV-1 (Pyke et al., 2016) offer ample evidence, following five decades of surveillance vacuum that at least three different lineages of sylvatic DENV are currently circulating in Southeast Asia suggesting that DENV has been maintained continuously in its zoonotic reservoir over the last 4 decades. Moreover, isolation of strain DKE-121 and the sylvatic DENV-1 described in Pyke et al extend the

documented geographic range of sylvatic DENV circulation [reviewed in (Vasilakis et al., 2011)] to include Borneo, the world's third largest island.

Serologic Relationships of DKE-121 with other Dengue Serotypes

Calisher and colleagues (Calisher et al., 1989) defined quantitative serologic criteria for the definition of a DENV serotype as a 4-fold or greater difference between homologous and heterologous neutralizing antibody titers. To serotype strain DKE-121, in collaboration with Dr. Tesh, curator of the World Reference Collection of Emerging Viruses and Arboviruses (WRCEVA), antigens as well as hyperimmune mouse ascitic fluids (HMAF) to the prototypic viruses of each of the 4 DENV serotypes (DENV-1 Hawaii; DENV-2 NGC or 16681; DENV-3 H87; DENV-4, H241) and to DKE-121 were generated using established methodologies (Vasilakis, Widen, et al., 2013). The cross-neutralization data with shown in Table 2.3 indicate a 4-fold difference in cross-neutralization between homologous and heterologous neutralizing antibody titers. Although several DENV studies (Vasilakis, Durbin, et al., 2008; Williams et al., 2014) use lower reduction percentages to report titers (e.g. $PRNT_{60}$), here a 80% endpoint was used to estimate conservatively *in vivo* protection. However, limited cross-reactivity among some of the serotypes is observed underlying the presence of cross-neutralizing epitopes, which are thought to play a role in the observed pathogenicity of heterologous infections in nature.

Table 2.3: Cross-neutralization titers of prototype strains of DENV-1-4 serotypes and the novel DENV strain DKE-121 using mouse hyperimmune sera (MIAF) raised against homologous DENV-1-4 and DKE-121 antigens. Highlighted in yellow are the homologous neutralization titers.

Virus type (strain)	Serum [a, b]				
	DENV-1	DENV-2	DENV-3	DENV-4	DKE-121
DENV-1 (Hawaii)	640	20	160	20	40
DENV-2 (NGC)	<20	1280	160	<20	80
DENV-3 (H87)	<20	<20	80	<20	<20
DENV-4 (H241)	<20	<20	<20	320	40
DKE-121	<20	<20	<20	<20	640

[a] $PRNT_{80}$

[b] Mouse hyperimmune sera (MIAF) raised against homologous DENV1-4 and DKE-121 antigens

The next step in the characterization was to serotype DKE-121 against a panel of well-characterized monotypic human sera. A panel of 12 monotypic DENV sera and 4 naïve sera were obtained from Dr. Rick Jarman of Walter Reed Army Institute of Research (WRAIR), Viral Diseases Branch. The neutralization assays were performed using the prototypic viruses of each of the 4 DENV serotypes (DENV-1 Hawaii; DENV-2 NGC or 16681; DENV-3 H87; DENV-4, H241) and DKE-121. As above, serotype specific MIAFs were used for the development of the assay and an 80% endpoint to estimate conservatively *in vivo* protection. The cross-neutralization data with shown in Table 2.4 indicate a 4-fold difference in cross-neutralization between homologous and heterologous neutralizing antibody titers. Surprisingly, DKE-121 is neutralized as equally as DENV-4 by its homotypic sera, suggesting that DKE-121 and DENV-4 viruses share antigenic epitopes with strong neutralizing potential. Based on the close genetic (Fig. 2.1) and evolutionary (Fig. 2.2) relationships of DKE-121 with DENV-4 viruses it's likely

that DKE-121 shares a close antigenic relationship to DENV-4, similar to DENV-1 to its sister clade of DENV-3.

Table 2.4: Cross-neutralization titers of prototype strains of DENV-1-4 serotypes and the novel DENV strain DKE-121 using a well-characterized panel of monotypic DENV-1-4 human sera obtained from WRAIR. Highlighted in yellow are the homologous neutralization titers.

	DENV						
	DENV-1 Hawaii	DENV-2 NGC	DENV-3 H87	DENV-4 H241	DENV-4 LF32	DENV-4 1120	DKE-121
Naïve Serum	<20	<20	<20	<20	<20	<20	<20
Naïve Serum	<20	<20	<20	<20	<20	<20	<20
Naïve Serum	<20	<20	<20	<20	<20	<20	<20
DENV-1 Serum	20	<20	<20	<20	<20	<20	<20
DENV-1 Serum	<20	<20	<20	<20	<20	<20	<20
DENV-1 Serum	80	<20	<20	<20	<20	<20	<20
DENV-2 Serum	<20	<20	<20	<20	<20	<20	<20
DENV-2 Serum	<20	<20	<20	<20	<20	<20	<20
DENV-2 Serum	<20	80	<20	<20	<20	<20	<20
DENV-3 Serum	80	<20	80	<20	<20	<20	<20
DENV-3 Serum	<20	<20	40	<20	<20	<20	<20
DENV-3 Serum	<20	<20	<20	<20	<20	<20	<20
DENV-4 Serum	<20	<20	<20	80	<20	<20	20
DENV-4 Serum	<20	<20	<20	160	20	<20	40
DENV-4 Serum	<20	<20	<20	20	<20	<20	<20

Antigenic Cartography of the Novel Dengue Virus

Antigenic cartography is a powerful tool that positions viruses and antisera as points on a map. It is based on the simple principle their position on the map is derived from the corresponding neutralization titer in the tabulated data. As a result, this method exploits the host response variation to determine their location (sera and viruses) on the map by measuring angles to it (e.g. location of antiserum) from known points at either

end of a fixed baseline, rather than measuring distances to the point (e.g. location of antiserum) directly. Thus, in principle cartography optimally triangulates the map, and as such it reduces measurement errors by measuring each virus against multiple antisera and vice versa and in the process accurately interprets inherent contradictions in the dataset.

Recently, the paradigm of antigenically-homogenous serotypes has shifted with the discovery that, although geographically and spatiotemporally diverse DENVs cluster in four genetically into distinct serotypes, antigenically they do not cluster in the same manner (Katzelnick et al., 2015). Likewise, as described earlier (see Fig. 2.2 and Fig. 2.3A) the novel DENV strain DKE-121, is quite divergent from the geographically and spatiotemporally diverse DENVs that cluster into four genetically into distinct serotypes (Fig. 2.3B).

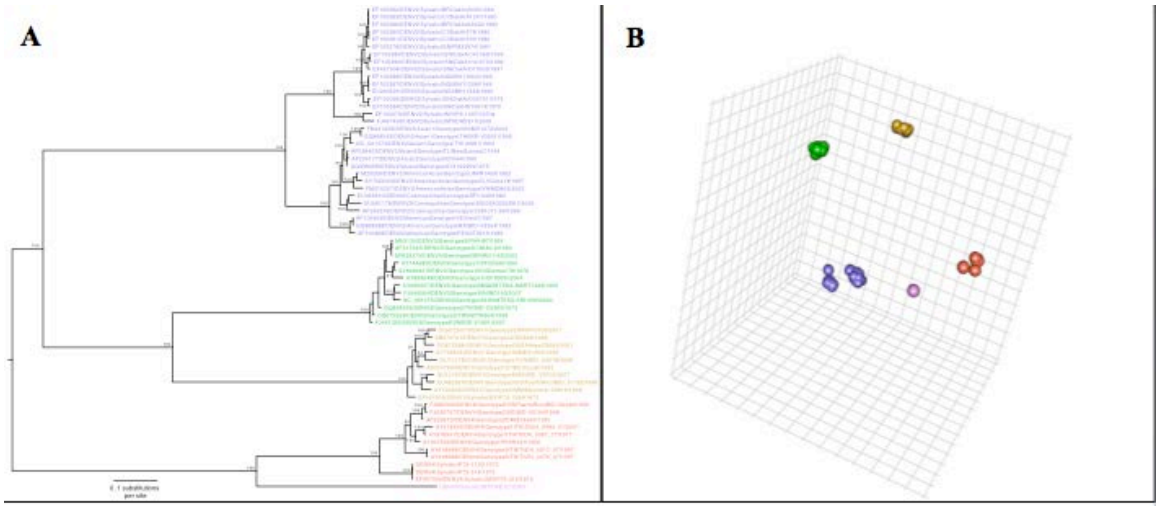

Figure 2.3: Genetic analyses of the DENV panel (n=64). (A) Phylogenetic tree showing the evolutionary relationships of DENV ORFs. (B) Amino acid map of DENV E protein sequences (493 to 495 amino acids in length). The total amino acid differences between pairs of E sequences correspond to distances between points on the geometric display. Blue – DENV-2; Green – DENV-3; Orange – DENV-1; Red – DENV -4; Mauve – DKE-121.

Therefore, further investigation of DKE-121 with a panel of monotypic human and NHP sera (provided by Drs. Jarman, WRAIR, and Whitehead, NIAID, respectively) was carried out. The neutralization assays were performed using the prototypic viruses of each of the 4 DENV serotypes (DENV-1 Hawaii; DENV-2 NGC or 16681; DENV-3 H87; DENV-4, H241) and DKE-121. As described above, serotype specific MIAFs were used for the development of the assay, but using a 50% endpoint to estimate *in vivo* protection. Neutralization titers were estimated as the intercept of a two-parameter logistic regression (top, or 'no neutralization' constrained to 1, bottom, or "full neutralization" constrained to 0, all with estimated slope). 'No neutralization' is defined as 2*(value set for 50% neutralization in raw data). For all data sets, the neutralization curves were estimated from all plaque counts simultaneously (so if duplicates, both values included in logistic regression). Titers estimated by the logistic regression that fall between 10 and 20 are included as their estimated value; titers estimated from the logistic regression that are less than 10 are set to <10, and titers where the logistic function could not estimate a curve are set to <20. Titers that are higher than the measured dilutions are set to a two-fold above the highest measured titer. Fig. 2.4A shows that that DKE-121 is antigenically distinct from DENV-4 and is as different from human DENV-4 viruses as other sylvatic DENV-4, and is less well neutralized by DENV-4 antisera than the human DENV-4 viruses. Fig. 2.4B indicates that antisera raised against DENV-4 recognize DKE-121, but that DKE-121 does not induce a response that consistently neutralizes DENV-4 better than other DENV types. DKE-121 is less well neutralized by DENV-4 antisera than the human DENV-4 viruses.

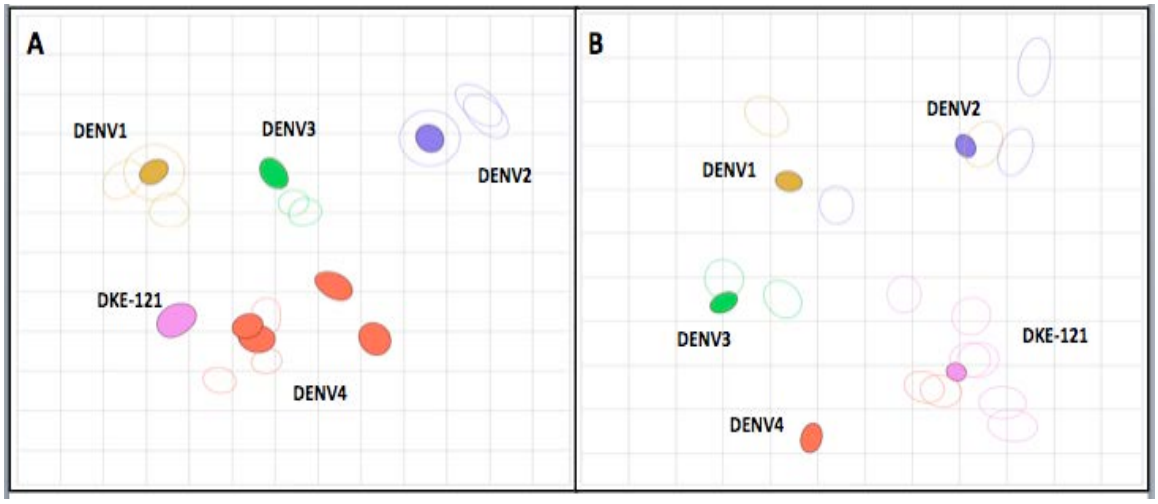

Figure 2.4: Antigenic relationships of DKE-121. (A) Antigenic relationships of DKE-121 to a monotypic panel of human DENV sera. (B) Antigenic relationships of DKE-121 to a monotypic panel of NHP DENV sera. Each unit of antigenic distance (length of one grid square side, measured in any direction) is equivalent to a two-fold dilution in the neutralization assay. Each antiserum (open shape) and virus (closed shape) is colored according to the infecting genetic type. The size and shape of each point is the confidence area of its position.

Collectively, the phylogenetic relationship to sylvatic DENV-4 lineages (Fig. 2.2), the cross-neutralization data shown in Tables 2.3 - 2.4 and the antigenic cartography to monotypic human and NHP sera (Fig. 2.4), indicate that DKE-121 is the prototype of a sylvatic DENV-5 serotype. Following the pioneering work of Rudnick on sylvatic DENV ecology in Malaysia in the 1960s (A. Rudnick, 1965; A Rudnick, 1986; A. Rudnick, Marchette, & Garcia, 1967) our knowledge of these viruses, and indeed of most sylvatic arboviruses in Asia, has been gleaned from serendipitous isolations from clinical cases. Indeed, the discovery of DENV-5 is another example of a serendipitous event. While spillover of sylvatic DENV in Malaysia, resulting in severe forms of dengue disease, has recently been documented (Cardosa et al., 2009; Pyke et al., 2016), there have been no systematic field studies of sylvatic DENV in its "cradle of emergence" in the last forty

years. However, the Asian sylvatic DENV-2 cycle has spawned a geographically discontinuous sylvatic cycle including West Africa, and the Vasilakis Lab and collaborators have been studying this system intensively over the last ten years with a combination of field, lab and modeling approaches (B.M. Althouse et al., 2015; Diallo et al., 2014; Hanley et al., 2014). The fieldwork in Senegal in collaboration with the Institut Pasteur has revealed that sylvatic DENV amplifications in primatophilic *Aedes* mosquitoes occur at ca. 8 year intervals, punctuated by "silent" periods during which the virus is not detectable in these mosquito vectors (B. M. Althouse et al., 2012). Proximity to large forests influences the distribution of putative sylvatic DENV vectors as well as the distribution of sylvatic arboviruses. Moreover, the Vasilakis laboratory studies have shown that, although sylvatic DENVs occupy a distinct ecological niche, these viruses do not require further adaptation to humans or peridomestic vectors to emerge into the human-endemic transmission cycle (Cardosa et al., 2009; Vasilakis, Shell, et al., 2007; Vasilakis, Tesh, et al., 2008). Additionally the Vasilakis Lab has demonstrated that the emergence of sylvatic DENV-1-4 strains into the human cycle would be constrained by homotypic immunity produced by natural infection with the homologous serotype in the human-endemic cycle, or by vaccination with the homologous serotype (Durbin et al., 2013; Vasilakis, Durbin, et al., 2008). However this last point must now be subject to a significant caveat – that the canonical DENV types are antigenically diverse, with major implications for their dynamics of immunity, evolution and vaccine development.

However in DENV, unlike most other arboviruses, spillover led to emergence into human transmission cycles, transmitted primarily by the domestic mosquito *Aedes aegypti* (Weaver, 2013). Phylogenetic analysis (Fig. 2.2), clearly shows that each of the 4

human-endemic serotypes emerged independently from the homologous serotype in a sylvatic cycle (Gubler, 1997; Holmes & Twiddy, 2003; Vasilakis et al., 2011; Vasilakis & Weaver, 2008; Wang et al., 2000) demonstrating the facility of DENV to jump between primate hosts. As described above, there is abundant ecological opportunity for sylvatic DENV to spill over into populations living near foci of transmission and ample evolutionary scope for emergence of novel DENV strains from the sylvatic cycle into human-endemic cycles. Moreover, longstanding faith in the ability of homologous antibody responses to protect against infection by all lineages within a serotype has recently been shaken by groundbreaking antigenic cartography on these viruses (Katzelnick et al., 2015). Despite the evident threat posed by the continued circulation of sylvatic DENV, its diversity in Asia, the nidus of viral radiation, has not been adequately characterized, nor has the frequency of sylvatic DENV spillover, or the burden of resulting disease, ever been quantified.

CHAPTER 3: Characterization of Dengue Virus Serotype 5, a Newly Emerged Dengue Virus in Non-Human Primates

INTRODUCTION

Dengue virus (DENV) belongs to the *Flavivirus* genus of the *Flaviviridae* family. Historically, DENV has been classified into four antigenically distinct serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) that are genetically related (Calisher, Karabatsos et al. 1989). Each serotype is sustained in nature in two transmission cycles, which are ecologically and evolutionary distinct (Rudnick 1986). In the human transmission cycle, domestic and peridomestic *Aedes* spp. (*Aedes aegypti* and *Ae. albopictus*) are important transmission vectors and humans are the only reservoir and amplification hosts for the virus (Hanley and Weaver, 2008). In the sylvatic cycle, various non-human primates (NHPs) species and arboreal *Aedes* spp. mosquitoes have been implicated as hosts and vectors of DENV transmission, respectively (Rudnick 1986). Evidences demonstrated that all sylvatic serotypes circulate concurrently in the forests of Southeast Asia (Rudnick 1965; Rudnick 1986; Saluzzo, Cornet et al. 1986), but only sylvatic DENV-2 is present in West Africa (Saluzzo, Cornet et al. 1986; Rodhain 1991; Diallo, Ba et al. 2003; Diallo, Sall et al. 2005). There is no documented sylvatic DENV transmission cycle in the Americas (Vasilakis and Weaver 2008; Vasilakis, Cardosa et al. 2011). Besides humans, NHPs represent the only natural mammalian host that is infected with DENV.

Several aspects of DENV infection and disease have been elucidated through studies using rhesus macaques as animal model (Sariol and White 2014). There is enough

indication that DENV isolated from the human cycle are able to replicate in rhesus macaques without the requirement of any adaptation, however the virus do not cause clinical disease in the infected NHP (Halstead and Palumbo 1973; Rodhain 1991; Peiris, Dittus et al. 1993). DENV infection via subcutaneous and intramuscular routes showed to successfully imitate the natural route of mosquito infection in NHPs. The period of viremia in NHPs and humans seem to be similar, although the replication level is inferior in NHPs (Marchette, Halstead et al. 1973; Zompi and Harris 2012). In fact, studies showed that dengue severe disease is not evident in NHPs and the viremia titer is significantly lower when compared to humans that were suffering of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Whitehead, Chaicumpa et al. 1970; Marchette and Halstead 1974; Halstead 1979; Rosen, Roseboom et al. 1985). The occurrence of hemorrhagic disease was only demonstrated in NHPs when a higher dose of DENV via intravenous route was employed (Onlamoon, Noisakran et al. 2010). NHPs from this particular study showed hemorrhagic signs including petechiae and hematomas, coagulopathy with increased D-dimers, a fibrin degradation product that is related to disseminated intravascular coagulation (DIC). On the other hand, no other signs of fever, anorexia or lethargy were described (Onlamoon, Noisakran et al. 2010).

Despite the low occurrence of pathology in NHPs infected with DENV (Halstead and Palumbo 1973; Koraka, Benton et al. 2007), they share several similarities with human disease. NHPs have naturally high levels of platelets, and thrombocytopenia is not frequently documented in these animals. However modest decrease in platelet numbers already has been reported in rhesus macaques (Halstead, Shotwell et al. 1973; Onlamoon, Noisakran et al. 2010). Other aspect related to human infection and also observed in

infected NHPs is a reduced DENV-specific antibody response during secondary homologous infection (Omatsu, Moi et al. 2011). Additionally, antibodies generated in primates during DENV infection are described to be highly cross-reactive against other closely related flaviviruses (Scherer, Russell et al. 1978).

Recently, a new strain of sylvatic DENV (DKE-121) was isolated from a human patient in Malaysia (Vasilakis, Mayer et al., in preparation). Serological studies and phylogenetic analysis demonstrated that this isolate represent a new DENV serotype (DENV-5) within the DENV serocomplex (Vasilakis, Mayer et al., in preparation). The objective of the present study was to characterize DENV-5 infection in non-human primates, evaluating several aspects of the disease and the innate and adaptive immune response during homotypic and heterotypic infection.

MATERIALS AND METHODS

Cells

Vero cells were maintained at 37°C in Dulbecco's Minimal Essential Medium (DMEM) (GIBCO, Life Technologies, Carlsbad, CA) supplemented with 5% heat inactivated fetal bovine serum (FBS) (Hyclone, Thermo Scientific, Logan, UT) and 100U/mL of penicillin and 100μg/mL of streptomycin (P/S) (GIBCO). C6/36 mosquito cells were propagated at 28°C in DMEM supplemented with 5% FBS, 100U/mL and 100μg/mL of P/S and tryptose phosphate broth (TPB) (Sigma-Aldrich, Saint Louis, MO).

Viruses

Low passage DENV isolates (DENV-1, Hawaii; DENV-2, NGC; DENV-3, H87; DENV-4, H142; DENV-5, DKE121) were propagated in C6/36 cultures to obtain high

titer stocks. Cell supernatants were clarified from cellular debris by low-spin centrifugation (630 x g, 10 min at 4°C), stabilized with the addition of 1X SPG (2.18 M Sucrose, 0.038M KH_2PO_4, 0.072M K_2HPO_4 and 0.054M L-glutamate), aliquoted and stored at -80°C. If necessary, viruses were further concentrated through AMICON filters (membrane with NMWL of 100 kDa, Millipore) following the manufacturer's protocol and purified on a sucrose gradient. Viral titers were determined by focus-forming assay (FFA) as previously described (Vasilakis, Shell et al. 2007).

Animals

Young adult male rhesus macaques (*Macaca mulatta*) (Chinese genetic background) tested seronegative for flavivirus infections were used in this study. In the first experiment, one group of animals (n=3) was inoculated subcutaneously (s.c.) with $1x10^3$ focus-forming units (FFU) and the second group (n=3) with a dose of $1x10^5$ FFU of DENV-5 DKE-121. After 6 months all the animals were re-challenged with $1x10^5$ FFU of DENV-5 DKE-121 (homologous challenge). A second experiment (n=4) was conducted at the Caribbean Primate Research Center (CPRC). Rhesus macaques (Indian genetic background) were inoculated s.c. with a dose of $5x10^5$ FFU of DENV-5 DKE-121. After 6 months, one group (n=2) was re-challenged with a human DENV-4 strain (LF32) and the other group (n=2) with a sylvatic DENV-4 strain (P75-514) at the dose of $1x10^5$ FFU (heterologous challenge).

All animal work was reviewed and approved by the Institutional Animal Care and Use Committees (IACUC) with oversight of staff veterinarians at the AAALAC-approved Animal Resources Centers (ARC) at the University of Texas Medical Branch (UTMB) and at the Medical Sciences Campus, University of Puerto Rico (UPR).

Quantification of the viremia titer

Viremia was assessed by focus-forming assay (FFA) on VERO cells as previously described (Vasilakis, Shell et al. 2007) using serial dilutions of serum samples collected from the NHPs on days 1 to 10 post-infection (pi). The number of foci was determined by immunostaining using a DENV-specific polyclonal antibody that recognizes all DENV serotypes as previously described (Rossi, Nasar et al. 2012).

Natural mosquito infection

Ae. albopictus (Galveston colony) were maintained at 28°C with 80% relative humidity and a 12/12 (light/dark) hour photoperiod. Mosquitoes were allowed to feed for 5-10 minutes in the NHPs infected with DENV-5 during the viremic phase at days 2, 3, 5 and 6 p.i. Engorged mosquitoes were collected and maintained at 28°C for 14 days. After 14 days, bodies and legs of mosquitoes were dissected, homogenized usiang a TissueLyser (Qiagen, Valencia, CA) and inoculated in C6/36 cells to determine the presence of virus by FFA and immunostaining (Rossi, Nasar et al. 2012).

Plaque reduction neutralization test (PRNT) and immunohistochemistry (IHC)

PRNTs were performed in 24-well plates, similarly as previously described (Durbin, Mayer et al. 2013). Constant virus inoculum (1,500 FFU/mL) was mixed with an equal volume of 2-fold serum dilutions (1:10-1:2,560) and the mixture was incubated 1hr at 37°C. Then, 100 μL of the serum-virus mixture was placed into Vero cultures and incubated 1hr at 37°C. A 1.0 mL volume of 0.8% methycellulose in OPTIMEM-I (GIBCO) overlay was placed in each well and the plates were incubated at 37°C for 4-5 days depending on the virus phenotype. The cells were then fixed with acetone and methanol (1:1) solution and immunostained as described previously (Vasilakis, Shell et

al. 2007; Vasilakis, Fokam et al. 2008). The PRNT titers were scored as reciprocal of the highest dilution of serum that inhibited 80% of foci ($PRNT_{80}$).

Multiplex serum cytokine assay

Antibody-coated magnetic microbeads optimized for quantifying NHPs specific cytokines and chemokines (Novex, Life Technologies) were used according the manufacturer's instructions. The cytokines assayed included interferon (IFN)-γ, interleukin (IL)-2, IL-4, IL-10 and tumor necrosis factor (TNF)-α (5-Plex Panel), IL-8 (Singleplex) and monocyte chemotactic protein-1 (MCP-1) (Singleplex). The data were acquired on a Luminex® 200 IS™ machine equipped with Bio-Plex Manager Software (version 5.0) (Bio-Rad, Hercules, CA) to determine each cytokine concentration.

Enzyme-linked immunosorbent assay (ELISA)

DENV-specific IgM and IgG responses were measured by using anti-human capture ELISAs (InBios International, Seatle, WA or Focus Diagnostics, Cypress, CA) following the manufacturer's instructions. Serum levels of DENV NS1 antigen was also measured by an ELISA assay system for the detection of NS1 in human serum (InBios International or Focus Diagnostics, Cypress, CA) following the manufacturer's instructions.

Blood analyses and clinical chemistry

Animals from experiment one (homotypic challenge) were submitted under complete blood count (CBC) examination using whole blood collected at days -3, 0, 3, 6 and 28 post-infection. CBC was run using a Drew Scientific HEMAVET 950FS Hematology System machine. Analysis included white blood cells (WBC), neutrophils (NEUT), lymphocytes (LYMPH), monocytes (MONO), eosinophils (EO), basophils

(BASO), red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT) and blood platelets (PLT).

Clinical chemistry was conducted on total blood samples in both experiments using Abaxis VETSCAN VS2 machine. Analysis included lactase dehydrogenase (LDH), aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

RESULTS

Non-human primate (NHP) infection with DENV-5 (DKE-121) and homotypic challenge

Prior to infection, the rhesus macaques were assessed for general health status and any previous exposure to flaviviruses. All animals presented good health condition and were seronegative for all DENV serotypes and other flaviviruses including St. Louis encephalitis (SLEV), West Nile virus (WNV) and Japanese encephalitis virus (JEV). Six NHP used in this study were segregated into two groups. As the infectious dose during the transmission of DENV by mosquito bite is not known, one group of rhesus macaques was inoculated with a lower dose (10^3 FFU) and the other group with a higher dose (10^5 FFU) of DENV-5 DKE-121 isolate. The rhesus macaques were monitored during 4 weeks for clinical signs of dengue disease, including rash and hemorrhage. No clinical manifestations of the disease were observed in any of the rhesus macaques challenged with DENV-5, which is consistent with previous studies using similar challenge doses and different serotypes (Halstead, Shotwell et al. 1973). The body weight and temperature of the NHP also were monitored; however no significant changes were seen

during the time course of the study (Fig.3.1). NHP also did not present any changes in behavior.

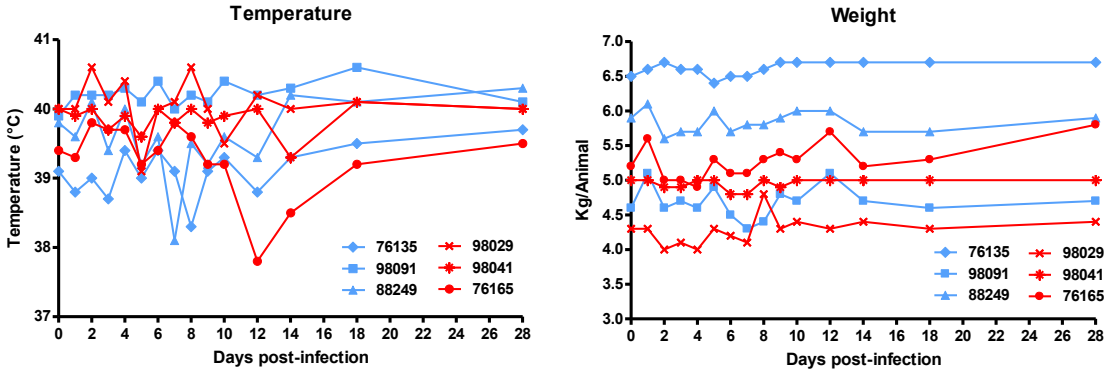

Figure 3.1. Changes in body weight and temperature of the NHP inoculated with DENV-5 DKE-121. Rhesus macaques inoculated subcutaneously with a dose of 10^3 FFU (blue lines) or a dose of 10^5 FFU (red lines) of DENV-5 DKE-121 isolate were monitored during four weeks.

To determine the viral load in the blood of NHP, samples were collected from days 1 to 10 pi. In the group inoculated with the lower dose of DENV-5, the viremia was detected starting at day 3 up to day 7 pi, with peak viremia titer near to 10^4 FFU/mL as determined by FFA (Fig. 3.2A). In the group inoculated with the higher dose, viremia was detected from days 1 to 6 pi, showing a biphasic pattern with peak titer reaching over 10^4 FFU/mL in one animal at day 4 after the infection (Fig. 3.2A). To further evaluate if the NHPs were infected with DENV, samples were tested in a capture ELISA to detect DENV NS1 presence in the serum of NHPs during the acute phase of infection. Five of six NHPs infected with DENV showed detectable NS1 in serum in at least 1 of the days tested (Fig.3.2B). Some of NHPs showed detectable NS1 as early as 1 dpi, with prevalence of detection during the first week of infection. At day 12, NS1 detection started to decline and was completely cleared by day 18pi (Fig.3.2B). There was no correlation between the infectious dose and the level of NS1 protein detected.

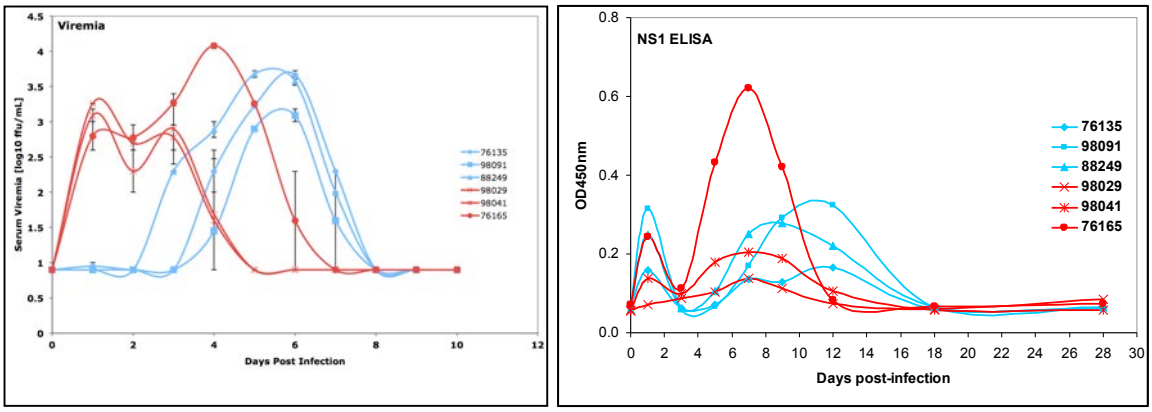

Figure 3.2. Viremia and DENV NS-1 secretion in the serum of the NHP inoculated with DENV-5 DKE-121. Viremia level tested by focus forming assay are shown in panel A. DENV NS-1 secretion tested by ELISA are shown in panel B. Rhesus macaques inoculated subcutaneously with a dose of 10^3 FFU (blue lines) or a dose of 10^5 FFU (red lines) of DENV-5 DKE-121.

After six months, the animals were re-challenged with DENV-5 (homotypic challenge) with an infectious dose of 10^5 FFU. After re-challenge, no clinical manifestations of the disease, nether viremia or DENV NS1 detection in the serum were detected in any of the animals.

Non-human primate (NHP) infection with DENV-5 (DKE-121) and heterotypic challenged with DENV-4

Four rhesus macaques were inoculated subcutaneously with DENV-5 DKE-121 strain with an infectious dose of $5x10^5$ FFU. Prior to infection, all NHP were assessed for general health status and any previous exposure to flaviviruses as described above. As expected, no clinical manifestations of the disease were observed in the rhesus macaques challenged with DENV-5. The body weight and temperature of the NHP also were

monitored; however no significant changes were seen during the time course of the study (Fig.3.3). NHP also did not present any changes in behavior.

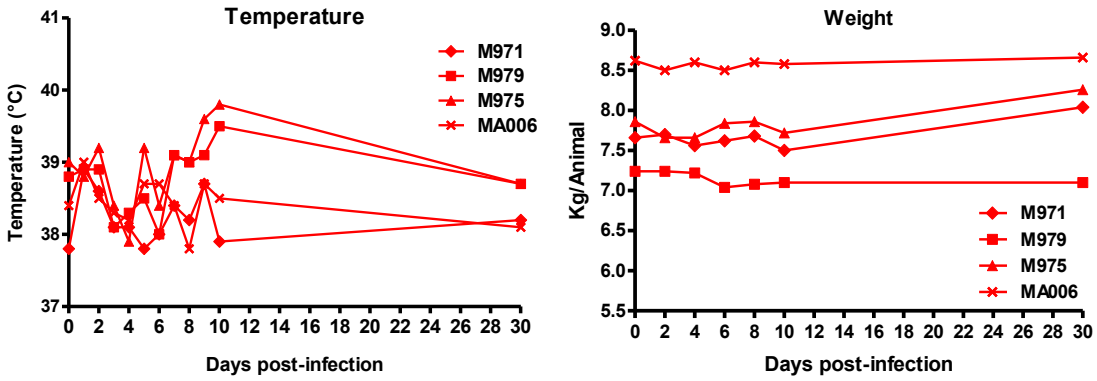

Figure 3.3. Changes in body weight and temperature of the NHP inoculated with DENV-5 DKE-121. Rhesus macaques inoculated subcutaneously with a dose of $5x10^5$ FFU of DENV-5 DKE-121 isolate were monitored during four weeks.

Viremia was detected in only one animal at day 5 pi (Fig.4.4A). Likewise, NS1 was detected in the same animal during the acute phase of infection starting at day 5 pi and peak at day 6 pi, following with a gradual decline during the course of infection (Fig.4.4B).

After six months, the animals were segregated in two groups and re-challenged with DENV-4 (heterotypic challenge). Two NHPs were inoculated subcutaneously with a human DENV-4 (LF32) and two with a sylvatic DENV-4 strain (P75-514) with an infectious dose of 10^5 FFU. No clinical manifestations of the disease were observed after challenge with any of those DENV-4 strains. Also, we were unable to detect any viremia by cell-based assays. Further, all animals were negative for DENV-NS1 secretion in the serum.

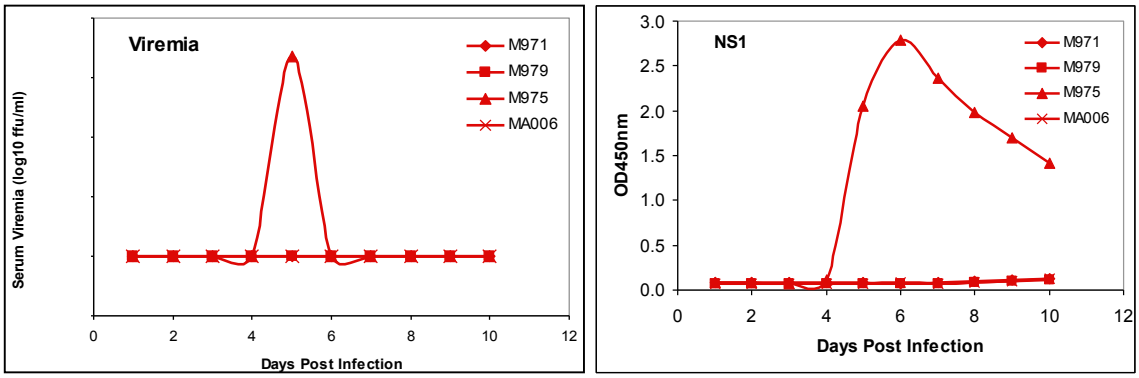

Figure 3.4. Viremia and DENV NS-1 secretion in the serum of the NHP inoculated with DENV-5 DKE-121. Viremia level tested by focus forming assay are shown in panel A. DENV NS-1 secretion tested by ELISA are shown in panel B. Rhesus macaques inoculated subcutaneously with a dose of $5x10^5$ FFU of DENV-5 DKE-121.

White bloods cells, platelet counts and serum biochemistry during DENV-5 infection

As DENV infection can cause alterations in cellular patterns and tissue damage, blood samples were collected in different time points with the objective of detect changes in blood cells population of DENV-5 infected NHP before (Fig. 3.5A) and after (Fig. 3.5B) homotypic challenge. A very modest leukopenia was observed in most of infected animals, however lowest levels of WBC were observed at 6 days pi in all animals and at days 1 and 12 post-homotypic challenges. Neutrophils levels were not evident decreased after DENV-5 infection, even though we observed some fluctuation among the course of infection and had a slightly variation among all animals.

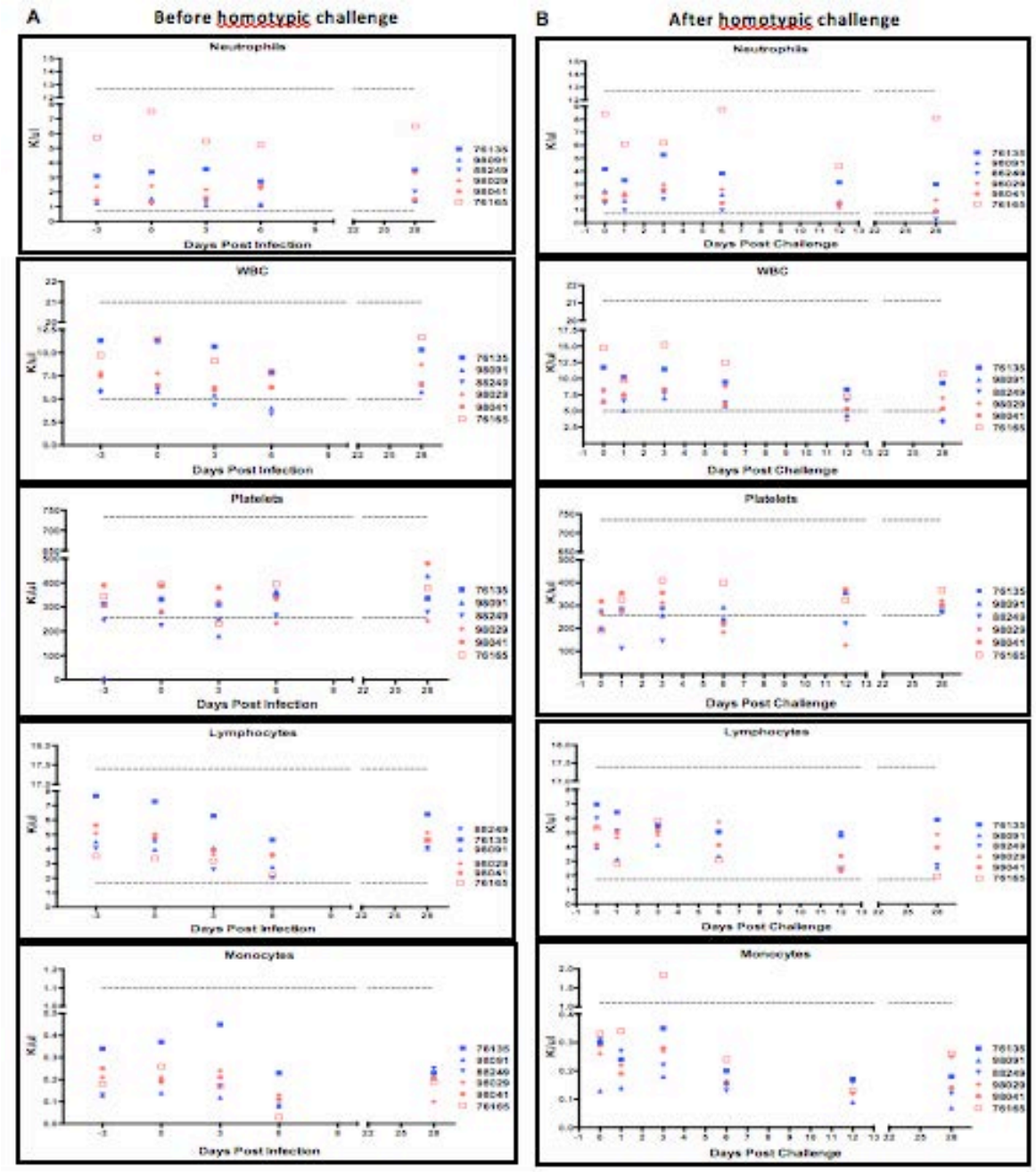

Figure 3.5. Changes in blood cell populations of the NHP inoculated with DENV-5 DKE-121. Blood were collected at the indicated time points and submitted to complete blood count (CBC) examination. (A) CBC values after primary DENV-5 infection. (B) CBC values after homotypic DENV-5 challenge. Rhesus macaques inoculated subcutaneously with a dose of 10^3 FFU (blue shapes) or a dose of 10^5 FFU (red shapes) of DENV-5 DKE-121.

Blood samples were also analyzed at 3 days prior infection and at day 3, 6 and 28 post-infection to measure liver enzymes levels. In the first experiment any alteration in liver enzymes after DENV-5 infection was detected. However, in the second experiment increased levels of AST and ALT were detected in the serum of all four animals, suggesting that these animals had liver injury after inoculation with DENV-5 (Fig.3.6).

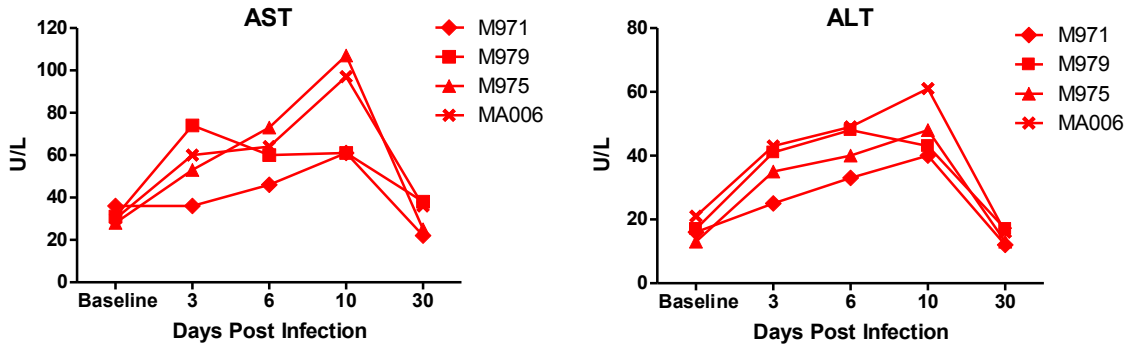

Figure 3.6. Changes in liver enzyme levels of the NHP inoculated with DENV-5 DKE-121. Blood were collected at the indicated time points and submitted to clinical chemistry examination. Rhesus macaques inoculated subcutaneously with a dose of $5x10^5$ FFU of DENV-5 DKE-121. Values after primary DENV-5 infection. AST: aspartate aminotransferase; ALT: alanine aminotransferase.

DENV-specific IgM antibody response

To further confirm if NHPs were infected with DENV-5 and evaluate the DENV-specific antibody response, IgM response was measured by ELISA in the serum collected at different time points, from days 0 to 150 pi and after challenge from days 28 to 150 post-challenge (see details in the figure legend). The serum IgM levels were increased starting at day 10 pi, with peak values between days 12 and 14 pi (Fig.3.7A). IgM levels still elevated at day 18, but started to decline reaching the initial levels by 28 dpi (Fig.3.7A). With the exception of one animal, the IgM levels between NHP infected with

the lower and higher doses were very similar. After the homotypic challenge at day 180 pi the IgM levels were not altered (Fig.3.7A).

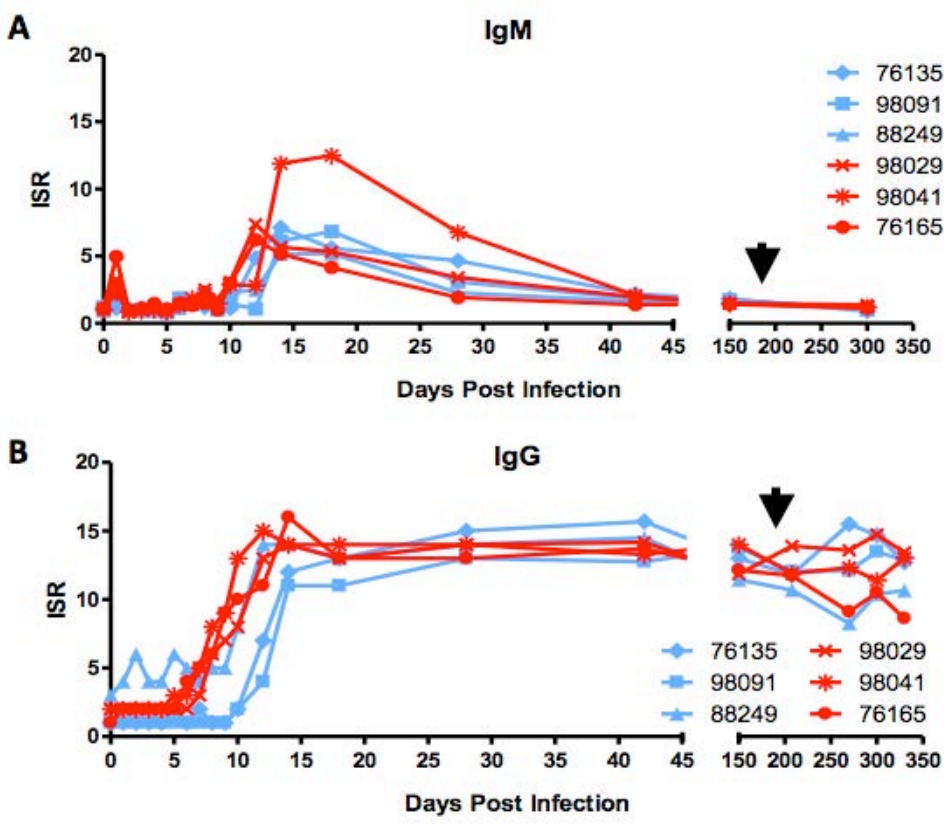

Figure 3.7. IgM and IgG antibody responses in the NHP inoculated with DENV-5 DKE-121 (homotypic challenge). Serum samples from the indicated time points were analyzed by using anti-human capture ELISA (InBios International, Seatle, WA) to detect DENV-specific IgM (A) or IgG (B) responses. Rhesus macaques inoculated subcutaneously with a dose of 10^3 FFU (blue lines) or a dose of 10^5 FFU (red lines) of DENV-5 DKE-121. The arrow indicate the homotypic challenge at 180 dpi. Immune Status Ratio (ISR) was calculated by dividing the DENRA (DENV recombinant antigen) OD with the NCA (normal cell antigen) OD.

In the second NHP study (heterotypic challenge) the IgM levels were very similar (Fig. 3.8A). However, when we measured IgM levels after heterotypic challenge with DENV-4, we observed a small peak in IgM levels very characteristic of a DENV secondary immune response in humans (TDR/WHO press 2009).

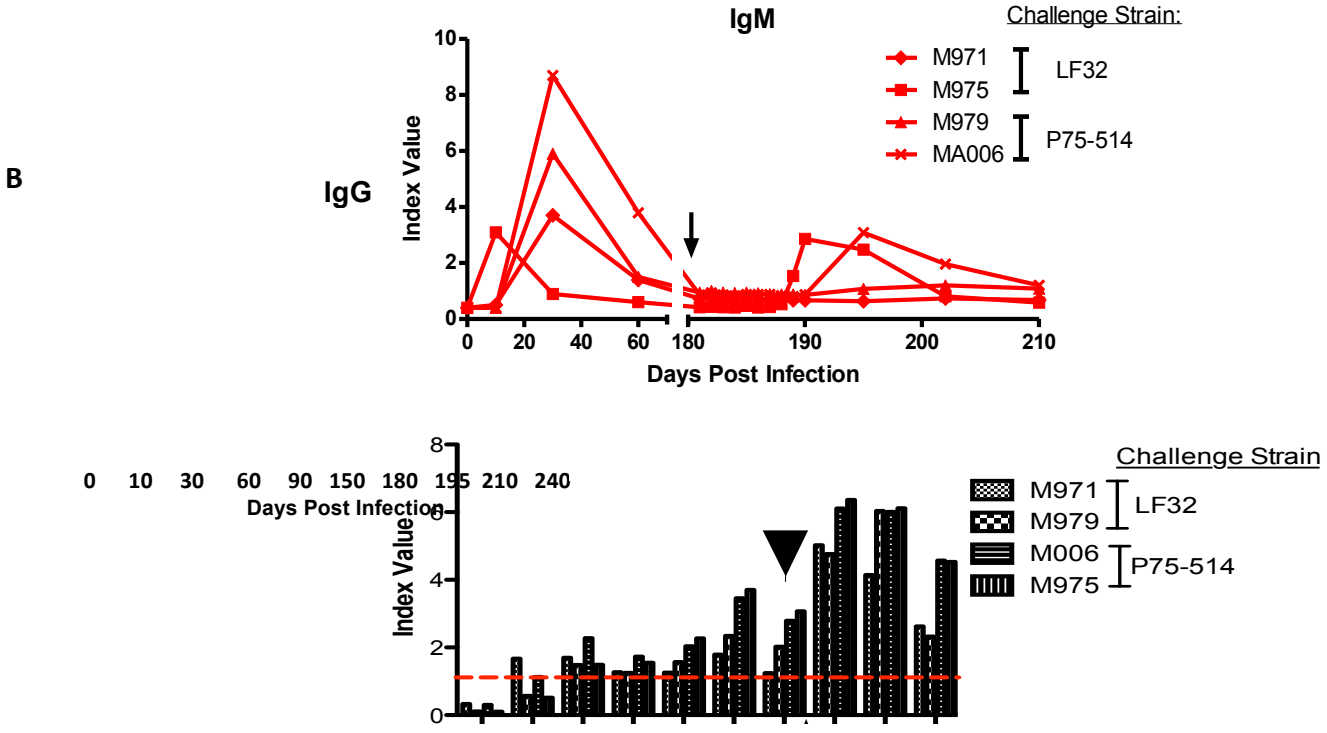

Figure 3.8. IgM and IgG antibody responses in the NHP inoculated with DENV-5 DKE-121 and re-challenged with DENV-4 (heterotypic challenge). Serum samples from the indicated time points were analyzed by using anti-human capture ELISA (Focus Diagnostics, Cypress, CA) to detect DENV-specific IgM (A) or IgG (B) responses. Rhesus macaques inoculated subcutaneously with a dose of $5x10^5$ FFU of DENV-5 DKE-121 and re-challenged with a human DENV-4 (LF32) or a sylvatic DENV-4 strain (P75-514) at the dose of 10^5 FFU. The arrow indicate the heterotypic challenge at 180 dpi.

DENV-specific IgG antibody response

The DENV-specific IgG response was evaluated using an ELISA test to measure IgG in the serum collected at several time points from days 0 to 150 post-infection and from days 28 to 150 after challenges (see details in the figure legend). The serum IgG levels started to rise at day 8 to 10 post-infection in the first experiment (homotypic

challenge) and kept a robust response until approximately one year after primary infection that correspond to the end of the experiment (Fig. 3.7B). Similar trend was observed when we measured IgG levels in our second NHP experiment after DENV-5 infection (Fig. 3.8B). After heterotypic challenge with two different DENV-4 strains (either human or sylvatic origin), a strong anamnestic response resembling a secondary infection in humans (TDR/WHO press 2009) was elicited in all four animals (Fig. 3.8B).

Detection of DENV neutralizing antibodies after primary DENV-5 infection and homotypic challenge

The neutralization activity of the serum of DENV-5 infected NHP was assessed by measuring the host neutralizing antibody response after primary DENV-5 infection and after the homotypic challenge. Neutralizing antibodies were detected in all six NHP infected with DENV-5 at day 28, 42, 150 and 180 dpi, with antibodies titers ranging from 160 to 1280 ($PRNT_{80}$) (Table 3.1). Six months after infection (180 dpi), the animals were re-challenged with DENV-5 (homotypic challenge). DENV neutralizing antibodies were detected in all animals at day 28 and 3, 5 and 6 months after the homotypic challenge, with levels ranging from 160 to 1280 (Table 3.1).

Table 3.1. Homotypic neutralization of serum samples from NHP inoculated with DENV-5 DKE-121 (homotypic challenge experiment).

Animal ID#	Days post infection							
	28	**42**	**150**	**180***	**208**	**270**	**330**	**360**
76135[a]	640[c]	640	320	320	320	160	320	160
98091[a]	320	640	1280	1280	1280	640	640	1280
88249[a]	160	640	640	640	640	640	320	320
98029[b]	640	640	1280	640	1280	1280	1280	1280
98041[b]	1280	640	640	640	320	320	320	320
76165[b]	640	640	320	320	320	320	320	320

[a] Monkey inoculated with 10^3 FFU; [b] Monkey inoculated with 10^5 FFU; [c] $PRNT_{80}$;
* Homotypic challenge at 180 dpi (10^5 FFU).

Cross-neutralization activity against the other DENV serotypes also was measured in the serum collected at the day of the homotypic challenge (180 dpi). As expected, any strong neutralization activity against the other DENV serotypes was observed. However, all animal sera tested against DENV-5 serotype demonstrated robust homotypic neutralization with more than fourfold increase compare to all other four serotypes (Table 3.2).

Table 3.2. Cross-neutralization titers of the serum from NHP inoculated with DENV-5 DKE-121 (homotypic challenge experiment).

Virus type (strain)	Serum*					
	76135[a]	98091[a]	88249[a]	98029[b]	98041[b]	76165[b]
DENV-1 (Hawaii)	<20[c]	20	<20	<20	NT	<20
DENV-2 (16681)	20	20	<20	20	NT	<20
DENV-3 (H87)	<20	<20	<20	<20	NT	<20
DENV-4 (H241)	20	80	40	20	NT	40
DENV-5 (DKE-121)	320	1280	1280	640	NT	320

[a] Monkey inoculated with 10^3 FFU; [b] Monkey inoculated with 10^5 FFU; [c] $PRNT_{80}$;
* Non-human primate serum at 180 days post infection; NT: not tested.

In the second NHP experiment a strong neutralization activity ($PRNT_{80}$) against DENV-5 serotype was also detected at day 30, 60, 90 and 180 pi (Table 3.3). Cross-neutralizing antibodies against the other DENV serotypes were also measured, showing only a slightly cross-neutralization activity against DENV-4 and no levels of neutralizing antibodies against DENV-1, DENV-2 or DENV-3 (Table 3.3). The level of neutralizing antibodies against DENV-5 (DKE-121) presented an increase during the time course of the infection with titers ranging from 160 to 320 at day 180 pi (Table 3.3). The heterotypic challenge with DENV-4 strains promoted the induction of strong neutralization titers in all NHP (Table 3.4).

Table 3.3. Cross-neutralization titers of the serum from NHP inoculated with DENV-5 DKE-121[a] (heterotypic challenge experiment).

Virus type (strain)	30 days post infection				60 days post infection				90 days post infection				180 days post infection*			
	M971	M979	M975	MA006	M971	M979	M975	MA006	M971	M979	M975	MA006	M971	M979	M975	MA006
DENV-1 (Hawaii)	<20[c]	20	40	20	20	20	20	20	20	20	20	20	20	20	20	20
DENV-2 (NGC)	<20	<20	<20	20	<20	<20	20	20	<20	<20	<20	20	<20	<20	20	20
DENV-3 (H87)	20	20	40	20	<20	20	20	20	20	20	20	20	<20	<0	20	20
DENV-4 (H241)	80	40	40	80	40	80	160	160	40	80	80	80	40	80	160	160
DENV-5 (DKE-121)	160	40	40	320	160	320	320	320	320	320	160	320	160	320	320	320

[a] Monkeys inoculated with $5x10^5$ FFU; [c] $PRNT_{80}$; * Day of heterotypic challenge; [c] $PRNT_{80}$.

Table 3.4. Cross-neutralization titers of the serum from NHP inoculated with DENV-5 DKE-121[a] and re-challenged with DENV-4 LF32[b] or P75-514[b] strains (heterotypic challenge experiment).

	DENV-5 (DKE-121)				DENV-4 (p73-1120)				DENV-4 (p75-514)				DENV-4 (LF32)			
Animal ID#	Days post re-challenge				Days post re-challenge				Days post re-challenge				Days post re-challenge			
	10	15	22	30	10	15	22	30	10	15	22	30	10	15	22	30
M971[d]	160[c]	160	160	640	80	80	40	160	160	80	40	160	80	80	80	160
M979[d]	640	320	320	320	160	160	160	160	160	160	160	160	320	320	160	160
M975[e]	≥1280	≥1280	≥1280	≥1280	640	≥1280	≥1280	≥1280	≥1280	≥1280	≥1280	≥1280	640	≥1280	≥1280	≥1280
MA006[e]	320	≥1280	≥1280	≥1280	160	≥1280	≥1280	≥1280	320	≥1280	≥1280	≥1280	160	≥1280	≥1280	≥1280

[a] Monkeys inoculated with $5x10^5$ FFU; [b] Monkeys re-challenged with 10^5 FFU on day 180 post primary infection; [c] $PRNT_{80}$; [d] Monkeys re-challenged with the human DENV-4 strain (LF32); [e] Monkeys re-challenged with the sylvatic DENV-4 strain (P75-514).

Serum cytokine changes after primary DENV-5 infection and after homotypic and heterotypic challenge

The profile of cytokine and chemokine response to DENV-5 serotype was characterized after primary infection and after both homotypic and heterotypic challenges. Serum levels of cytokines and chemokines were measured using multiplexed microsphere protein-based assay. The cytokines and chemokines tested included IL-8, IL-2, IL-4, IL-10, IFN-γ, MCP-1, and TNF-alpha. After the primary infection with DENV-5 all animals from the first experiment (homotypic challenge) did presented IL-2, IL-10 and TNF-alpha below the levels of detection (Fig. 3.9). IL-4 levels were not elevated during the acute phase of primary DENV-5 infection. However, IFN-gamma, IL-8 and MCP-1 levels were slightly increased in most of the NHP (Fig.3.9). After homotypic challenge, IL-10 and TNF-alpha were also below the level of detection in all six NHP and two out of six animals had IL-2 secretion in the serum between days 8 to 10 post challenge (Fig.3.10). Similarly to the primary infection, IFN-gamma, IL-8 and MCP-1 have slight increased levels in some of the NHP after the homotypic challenge (Fig.3.10).

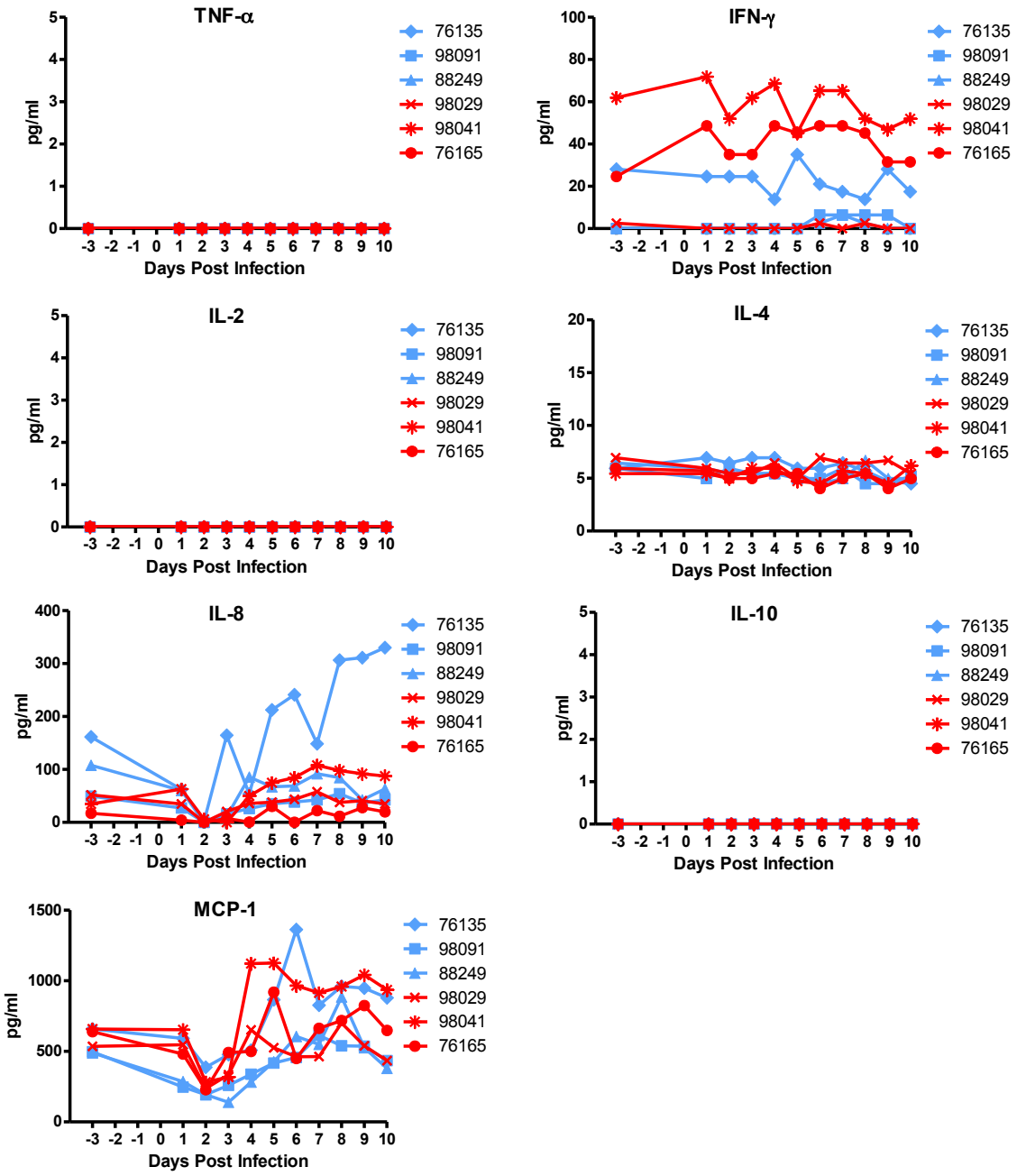

Figure 3.9. Cytokine and chemokine profile in the NHP inoculated with DENV-5 DKE-121 during primary infection (homotypic challenge experiment). Serum samples from the indicated time points were analyzed by using a multiplex cytokine assay. Rhesus macaques inoculated subcutaneously with a dose of 10^3 FFU (blue lines) or a dose of 10^5 FFU (red lines) of DENV-5 DKE-121.

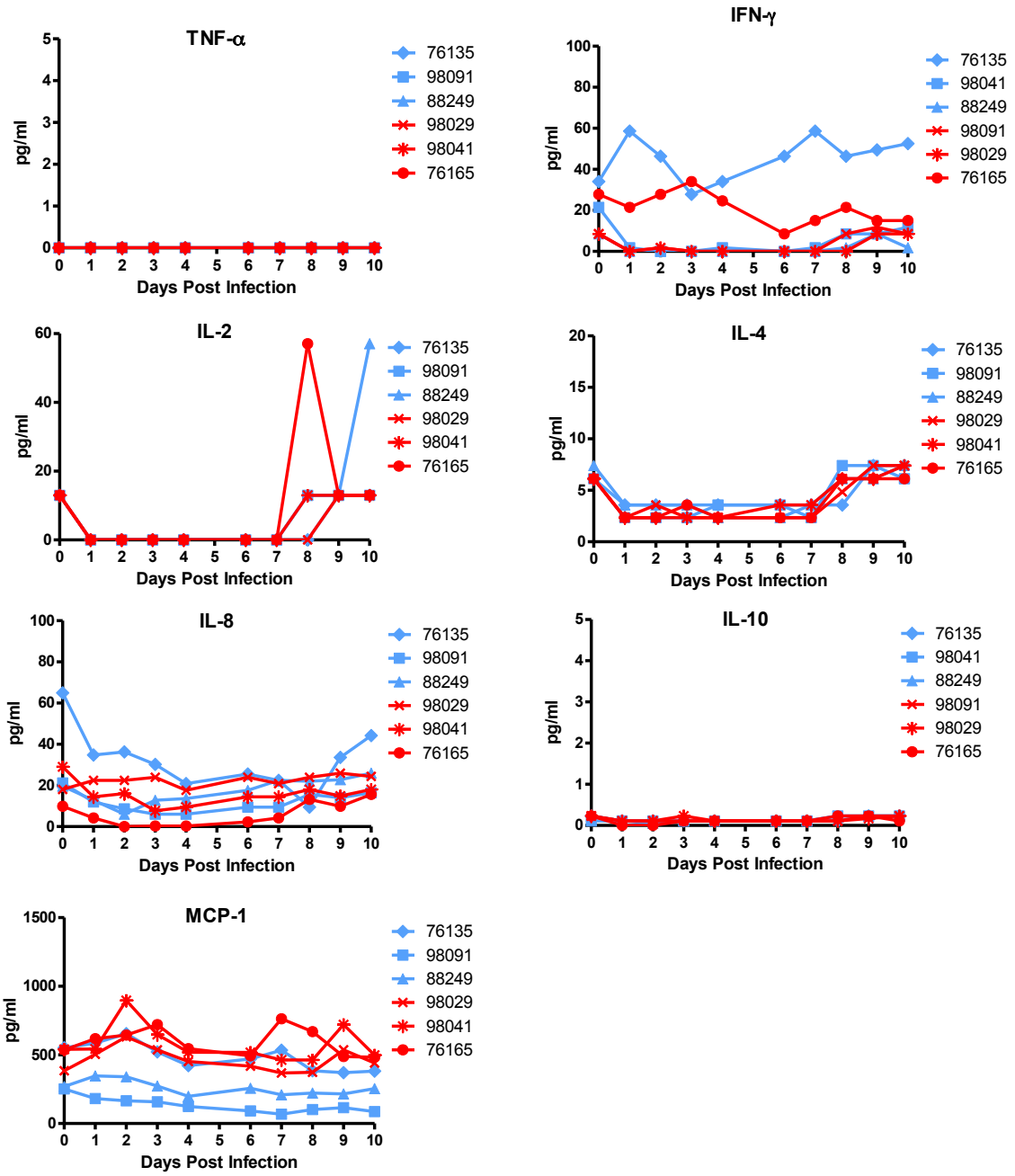

Figure 3.10. Cytokine and chemokine profile in the NHP inoculated with DENV-5 DKE-121 after homotypic challenge (180 dpi). Serum samples from the indicated time points after the homotypic challenge were analyzed by using a multiplex cytokine assay. Rhesus macaques inoculated subcutaneously with a dose of 10^3 FFU (blue lines) or a dose of 10^5 FFU (red lines) of DENV-5 DKE-121.

In the second experiment (heterotypic challenge) any IL-10 was detected during the primary DENV-5 infection (Fig.3.11) or after the challenge with DENV-4 strains (Fig.3.12). Although, with some level variation among the animals, IFN-gamma was detected in all four NHP after primary infection (Fig.3.11) and in two out of four NHP after heterotypic challenge with DENV-4 (Fig.3.12). Levels of TNF-alpha and IL-4 increased in all animals during the primary DENV-5 infection with a peak at days 3 and 4 after the infection that remained consistently high until day 10 post-infection, the last day they were measured (Fig. 3.11). However, no TNF-alpha or IL-4 were detected after the heterotypic challenge (Fig. 3.12). IL-2 was detected in three out of four animals after primary DENV-5 infection (Fig. 3.11), however after heterotypic challenge this cytokine was below level of detection in all animals (Fig. 3.12). Levels of IL-8 elevated after primary infection (Fig. 3.11), however after heterotypic challenge this cytokine had very modest secretion only in one NHP (Fig. 3.12). MCP-1 levels were slightly increased before and after heterotypic challenge (Fig. 3.11 and 3.12, respectively).

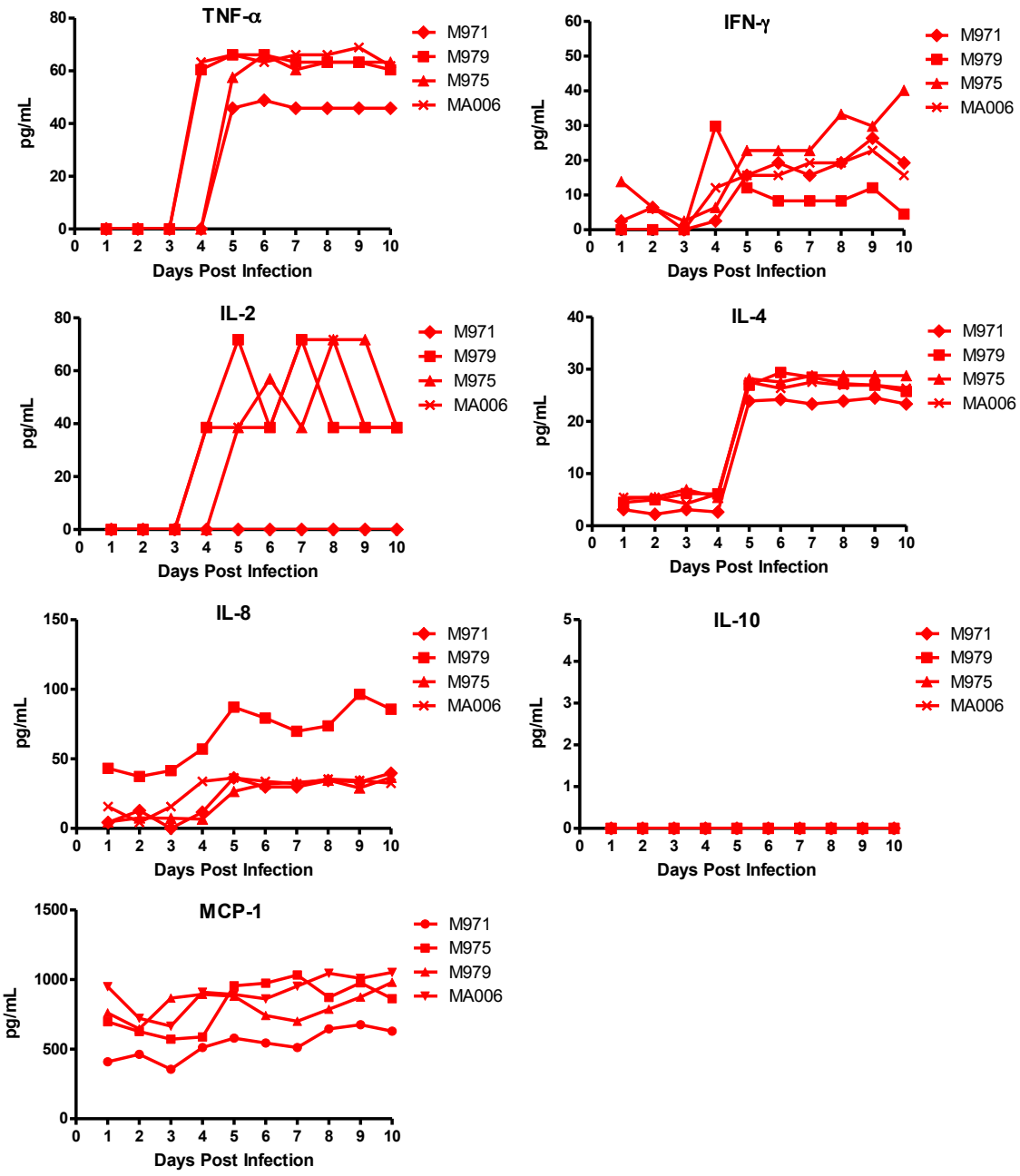

Figure 3.11. Cytokine and chemokine profile in the NHP inoculated with DENV-5 DKE-121 during primary infection (heterotypic challenge experiment). Serum samples from the indicated time points were analyzed by using a multiplex cytokine assay. Rhesus macaques inoculated subcutaneously with a dose of $5x10^5$ FFU of DENV-5 DKE-121.

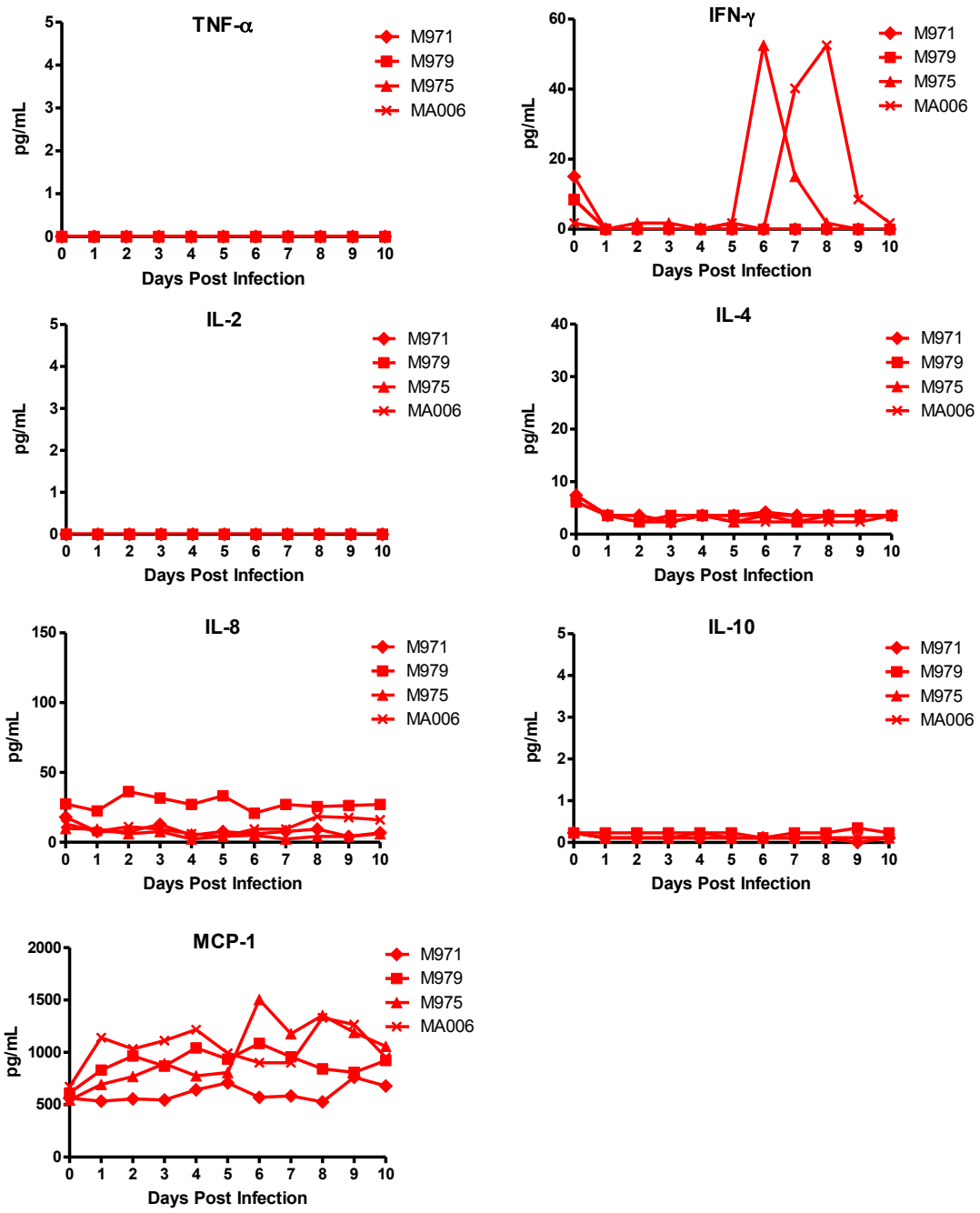

Figure 3.12. Cytokine and chemokine profile in the NHP inoculated with DENV-5 DKE-121 after heterotypic challenge. Serum samples from the indicated time points after the heterotypic challenge were analyzed by using a multiplex cytokine assay. Rhesus macaques were inoculated subcutaneously with a dose of $5x10^5$ FFU of DENV-5 DKE-121 and re-challenged at 180 dpi with a human DENV-4 (LF32) (monkeys #M971 and M979) or a sylvatic DENV-4 strain (P75-514) (monkeys #M975 and MA006) at the dose of 10^5 FFU.

DISCUSSION

Non-human primates (NHPs) are a suitable animal model for viremia and host immune response studies in dengue disease. However they have been described to not mimic acute dengue fever or dengue hemorrhagic fever and the occurrence of pathology is very limited (Clark, Onlamoon et al. 2013; Hickey, Koster et al. 2013). Despite this model does not present remarkable pathology and symptoms that can be correlated to human DENV infection, they still share a variety of characteristics to human disease. For example, the period of viremia, the occurrence of leukopenia (Onlamoon, Noisakran et al. 2010) and modest levels of platelets ((Halstead and Palumbo 1973; Onlamoon, Noisakran et al. 2010) had been described in some species of NHPs infected with DENV. Another observation in DENV infection such as the reduction of specific antibodies during the initial phase of a secondary exposure by a homologous DENV serotype in human patients has been described in studies using marmosets as NHP model (Omatsu, Moi et al. 2011). Additionally, similar to what happen in humans, infection of NHP by DENV has been reported to stimulate a robust innate immune response leading to activation of T, NK and NKT cells in marmoset's studies (Sariol, Munoz-Jordan et al. 2007; Yoshida, Omatsu et al. 2013). In first experiment (homotypic challenge)**,** virus was detected in the serum of all six rhesus macaques (Chinese genetic background) infected with DENV-5 serotype (Fig.3.2A). The viremia started at day 1 and extended until day 8 post-infection, with animals having some variation between the higher (10^5 pfu) and lower (10^3 pfu) virus dose. The peak of viremia was observed at day 4 post-infection for animals infected with the higher dose and at 6 days post-infection for ones infected with

the lower dose. These results are similar regarding the period of secreted virus in the blood and peak of viremia to studies in rhesus macaques where all DENV serotypes were used (Halstead and Palumbo 1973). Similarly, a more recent study in rhesus macaques infected with DENV has demonstrated that the peak of viremia was reached at day 5 post-infection and lasted until day 8 (Hickey, Koster et al. 2013). Another study using green monkeys (*Chlorocebus sabaeus*) also corroborate with the current data showing longer viremia in animals inoculated with a lower dose compared to a higher dose of DENV (Martin, Hermida et al. 2009). In the second experiment (heterotypic challenge), viremia was detected in only one animal at day 5 post-infection (Fig.3.4A). It is possible that the differences observed between the two experiments maybe the result of the particular stronger innate immune response, particularly the TNF-alpha response (Figure 3.11), which could result in the control of DENV replication and dissemination in these animals with a different genetic background.

Blood levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are affected in a variety of diseases including dengue. There are several reports of liver damage being mediated by DENV infection (Jessie, Fong et al. 2004; de Macedo, Nicol et al. 2006; Tristao-Sa, Kubelka et al. 2012). An evident increase of serum AST and ALT levels was observed in all rhesus macaques in experiment two (Indian genotype) after primary DENV-5 infection. This is consistent with previous reports in animals infected with all other four serotypes (Hickey, Koster et al. 2013). However, no alteration in AST and ALT were observed in the rhesus macaques of Chinese origin infected with DENV-5.

The infection of NHPs with the new DENV serotype resulted in a modest

decreased of lymphocytes and neutrophils in animals from the first experiment. The reduction of neutrophil levels associated with DENV infection has been described in studies from Singapore (Thein, Lye et al. 2014). Furthermore, dengue disease has also been described to caused lymphopenia and neutropenia in human patients from French Polynesia during a DENV-2 epidemic (Deparis, Roche et al. 1998). Similar results were observed in DENV studies using rhesus macaques inoculated with different DENV serotypes (Hickey, Koster et al. 2013). In the current study a slight decrease in monocyte levels were observed after DENV-5 inoculation, contrasting with other groups work, where the levels of monocytes were described to be elevated during DENV infection in human and NHPs (Hickey, Koster et al. 2013; Kwissa, Nakaya et al. 2014).

Antibodies are important elements in dengue disease considering they provide immunity that can last for life; likewise they are tightly related to development of severe disease. So, studies of DENV antibodies in NHP as animal models can elucidate several aspects of dengue pathogenesis. Primary infections induce strong IgM and IgG responses while heterotypic secondary infections results in a modest increase in the IgM levels and a robust IgG levels boost. However secondary exposition to same serotypes does not induce detectable IgM or IgG levels as result of a sterilizing immunity after the primary infection (TDR/WHO press 2009). In the current study, the levels of DENV specific IgM and IgG antibodies were measured after primary DENV-5 primary infection and after homotypic and heterotypic challenges with DENV-4 serotype. All animals did produce DENV-specific IgM and IgG antibody responses after the primary infection in both experiments, demonstrating that DENV-5 serotype was able to infect the animals. Similarly to previous studies in NHPs measuring IgM response against the other four

DENV serotypes, IgM antibodies were detected in the NHPs cohorts exposed to DENV-5 at least until 28 days post-infection (Koraka, Benton et al. 2007; Martin, Hermida et al. 2009; Hickey, Koster et al. 2013). Furthermore, the magnitude of IgM levels measured after the challenge with DENV-4 serotype indicated a typical heterotypic immune response, supporting that both viruses do not belong to the same serotype classification as suggested by phylogenetic analyses (Vasilakis, Mayer et al., in preparation). This fact is confirmed by the absence of IgM response after the homotypic challenge.

In addition, detection of IgG antibodies was presented in a robust level and lasted for at least 180 days post primary infection before the challenges. IgG levels remains without changes after the homotypic challenge; however they were boosted more than 3 times after the challenge with DENV 4 strains providing additional serological evidence in support of DKE-121 being a new serotype.

Serum from the NHPs inoculated with DENV-5 were tested for the ability to build a robust neutralization response before and after challenging them with homologous and heterologous viruses as well as if there was cross neutralization activity against the other DENV serotypes using a heterotypic neutralization panel. A strong homotypic neutralization response was observed in all animals after the primary infection supporting the IgG results. More relevant is the fourfold increase in the neutralizing titers against DENV-5 compared to DENV-4 serotypes at day 180 after the primary infection. Six months after infection is considered an accepted period of time to expect the cross-neutralizing antibodies fading while remaining only the specific neutralizing antibodies. This result confirms that the immune system of the NHP exposed to DKE-121 recognized this strain as a different serotype, even to the closest relate serotype, the DENV-4.

The severity of dengue disease has been associated with elevated pro-inflammatory cytokines and chemokines in individuals presenting symptoms of dengue hemorrhagic fever before and during the time of plasma leakage (Srikiatkhachorn and Green 2010). A slight increase in IFN-gamma and MCP-1 levels was detected before and after homotypic challenge with DENV-5. Likewise, the serum level of MCP-1 was slightly increased before and after the heterotypic challenge with DENV-4. Secretion of MCP-1 had being described to have a potential role in dengue pathogenesis. Previous studies have correlated the effect of MCP-1 in promoting alteration on vascular permeability of tight junctions on endothelial cells (Yamada, Takasaki et al. 2001; Stamatovic, Keep et al. 2003). Furthermore, chemokines such as MCP-1 is secreted in response to signals of proinflammatory cytokines and they are crucial in the recruitment of lymphocytes, monocytes and neutrophils to the site of inflammation (Loetscher, Pellegrino et al. 2001)**.** IL-8 also has detected in the serum of the NHPs, especially in the second experiment, after the primary infection with DENV-5. Several studies in human subjects had associated this cytokine to severe dengue (Raghupathy, Chaturvedi et al. 1998; Juffrie, van Der Meer et al. 2000). Some studies supported that his cytokine can act through the mechanism involving IL-1 and TNF-alpha induction (Hoffmann, Dittrich-Breiholz et al. 2002). Furthermore, there is strong evidence of its role in the alteration of the cytoskeleton and tight junctions of the microvascular endothelium, altering its permeability and playing a role in the vascular leakage during the severe dengue disease in humans (Talavera, Castillo et al. 2004). IL-4 had slightly elevated levels after the homotypic challenge with DENV-5. Interestingly, all animals from the second experiment (heterotypic challenge) had a sustained increased in IL-4 levels only after the

primary DENV-5 infection. This data imply that DENV-5 rather then DENV-4 serotype is a trigger for the secretion of IL-4 in this NHP dengue model. Similar trend was observed when we analyzed IL-2 levels in the serum, as this cytokine was detected after primary infection with DENV-5 and after the homotypic challenge only, and no detection was observed after DENV-4 challenge. The current data are in agreement with previous rhesus macaque experiments using all four DENV serotypes where variation in cytokine levels were observed among the different groups, indicating that certain serotypes trigger specific profile of cytokines, suggesting that are likely virus-related differences in the immune response (Hickey, Koster et al. 2013). Nevertheless, the data contrast with other studies, where no up-regulation of genes associated with severe dengue disease was detected in rhesus macaques infected with DENV-1 (Sariol, Munoz-Jordan et al. 2007). TNF-alpha was another cytokine that was found increased in the serum of the NHPs from the second experiment (heterotypic challenge) after the primary DENV-5 infection. TNF-alpha has been described to play a role in both protection and immunopathogenesis of dengue disease. After DENV infection, biological mediators including IL-6 and TNF-alpha are released and although they are important in the initiation and control of inflammatory and adaptive immune responses to clear the pathogen, their actions can also be frequently involved in lethal manifestation associated with vascular leakage and shock syndrome (Tracey and Cerami 1994; Iwasaki and Medzhitov 2010; Kawai and Akira 2011). In mice model, elevated levels of TNF-alpha in the serum were detected after DENV infection (Chen, Ng et al. 2015), which is in agreement with studies in dengue patients where elevated TNF-alpha and IL-6 were detected (Hober, Poli et al. 1993; Pinto, Oliveira et al. 1999; Restrepo, Isaza et al. 2008). In addition the antiviral role of

TNF-alpha have been documented (Hober, Poli et al. 1993; Gagnon, Mori et al. 2002). It can be suggested that the lack of viremia after primary infection with DENV-5 in the second experiment in contrast with the detected viremia in the NHP in the first experiment maybe related to the early and high levels detected of that cytokine.

In summary, these studies demonstrated a systematic analysis of the infection and immune response against a new DENV serotype isolated from a human case in Malaysia. Using a NHP animal model, it was showed that the virus has the potential to be transmitted among NHPs and mosquitoes, which could result in the establishment of a sylvatic transmission cycle. The homotypic and heterotypic response was characterized, showing the antibody response against homologous and heterologous DENV strains. In addition, some aspects of the innate immune response, which is relevant to studies of DENV pathogenesis, were evaluated. Overall, the data presented in this study support the existence of a new dengue serotype and open more question about the future of an effective therapy or vaccine against dengue. Also this work confirms the value of the NHP as a suitable model to study different aspect of dengue virus, including infection and pathogenesis.

CHAPTER 4: Vector Competence of *Ae. aegypti* and *Ae. albopictus* mosquitoes for Dengue Virus Serotype 5, a Newly Emerged Dengue Virus

INTRODUCTION

The incidence and geographic range of dengue disease, including severe dengue disease such as dengue hemorrhagic fever (DHF) have increased dramatically in recent decades (Chen et al., 2005; Higa, 2011; Bhatt et al., 2013). Four serotypes of dengue virus (DENV-1-4) circulate in a human-endemic cycle in which Ae. aegypti is the primary vector, while Ae. albopictus and, to a lesser degree, Ae. polynensiensis play a secondary role. Each of the four human-endemic serotypes emerged independently from a sylvatic ancestor maintained in a transmission cycle between nonhuman primates (NHPs) and arboreal Aedes mosquitoes (Vasilakis, Cardosa et al. 2011). Recently, a fifth DENV serotype (DENV-5 DKE-121) has been isolated from a patient in the state of Sarawak, Malaysian Borneo who presented clinical symptoms compatible with dengue disease including high fever, headache, retro-orbital pain, myalgia, arthralgia and episodes of spontaneous gum bleeding (Vasilakis, Mayer et al., in preparation). DENV-5 DKE 121 is most closely related to sylvatic DENV-4 and clearly infected a human via spillover from the sylvatic cycle. Similarly, a unique strain of sylvatic DENV-1 has been isolated from a viremic patient who had visited the Malaysian rainforest of Brunei and returned to Australia with symptoms of DENV infection (Pyke, Moore et al. 2016).

Emergence of a fifth DENV serotype into the human-endemic cycle would have

profound implications for the development of diagnostics, therapeutics and vaccines. The rationale in the development of the current dengue vaccines requires simultaneous strong homotypic immune responses to all DENV serotypes, which has not been easy to be achieved. The introduction of one more serotype would complicate the production of polyvalent DENV vaccines that can prevent infection without the risk of severe disease enhancement following natural exposure to the virus.

The likelihood that DENV-5 will emerge into transmission among humans depends on the vector competence, i.e. the ability of a vector to support infection and dissemination, of *Ae. aegypti* and *Ae. albopictus* for the virus. The time period required for DENV, the extrinsic incubation period (EIP), ranges from 7 to 14 days depending on mosquito strain, virus genotype and environmental factors (Watts, Burke et al. 1987; Black, Bennett et al. 2002; Anderson and Rico-Hesse 2006; Salazar, Richardson et al. 2007; Lambrechts, Chevillon et al. 2009; Lambrechts 2010; Lambrechts, Paaijmans et al. 2011). DENV infects the midgut epithelial cells throughout receptor-mediated endocytosis and start to replicate in the first 2 days post-infection (dpi) (Bennett, Olson et al. 2002; Rey 2003; Mercado-Curiel, Black et al. 2008). As the infection progresses, the virus spreads to adjacent epithelium cells (Salazar, Richardson et al. 2007) and reaches a peak titer in the midgut between 7 to 10 dpi (Salazar, Richardson et al. 2007; Xi, Ramirez et al. 2008). DENV dissemination from the midgut throughout the body may occur through the tracheal system (Salazar, Richardson et al. 2007). Virus can be isolated from salivary glands as early as 3 dpi, with peak titers in 12 to 18 dpi (Salazar, Richardson et al. 2007). Upon completion of the EIP, DENV can be transmitted to a new host via saliva during feeding (Watts, Burke et al. 1987; Black, Bennett et al. 2002; Anderson and Rico-

Hesse 2006; Salazar, Richardson et al. 2007; Lambrechts, Paaijmans et al. 2011).

Vector competence for DENV is most often assessed by feeding mosquitoes on artificial blood meals spiked with designated concentrations of virus (Althouse and Hanley 2015). Using this method, multiple investigators have demonstrated a higher degree of salivary gland infection and transmission in *Ae. aegypti* mosquitos compared to *Ae. albopictus* (Chen, Wei et al. 1993; Vazeille, Rosen et al. 2003; Lambrechts, Scott et al. 2010). *Ae. aegypti* is also considered to be more efficient vector of DENV to humans than *Ae. albopictus* due differences in their feeding behavior; the former is anthropophilic and occurs primarily in urban environments while the latter has a broader host preference and occurs primarily in peri-urban and agricultural habitats (Rodhain and Rosen 1997; Bonizzoni, Gasperi et al. 2013; Takken and Verhulst 2013). However these differences may also make *Ae. albopictus* a particularly good bridge vector to mediate spillover of sylvatic DENV into humans. Another factor that differ between *Ae. aegypti* and *Ae. albopictus* is that *Ae. albopictus* is infected with a endosymbiotic bacteria *Wolbachia*, which is not present in *Ae. aegypti* (Kittayapong, Baisley et al. 2000; Sinkins 2004). The presence of this bacteria is described to protect mosquitoes against viral infections in nature (Hedges, Brownlie et al. 2008).

In the present study the vector competence of *Ae. aegypti* and *Ae. albopictus* fed on artificial bloodmeals containing either DENV-5 DKE-121 or one of two sylvatic or two human-endemic strains of DENV-4 was evaluated. Because artificial bloodmeals may underestimate vector competence (Althouse and Hanley 2015), the levels of infection and dissemination in *Ae. albopictus* that fed on viremic monkeys infected with DENV-5 DKE-121 also was tested.

MATERIAL AND METHODS

Cells and Viruses

Vero cells were maintained at 37°C in Dulbecco's Minimal Essential Medium (DMEM) (GIBCO, Life Technologies, Carlsbad, CA) supplemented with 5% heat inactivated fetal bovine serum (FBS) (Hyclone, Thermo Scientific, Logan, UT), 100U/mL of penicillin and 100μg/mL of streptomycin (P/S) (GIBCO). C6/36 mosquito cells were propagated at 28°C in DMEM supplemented with 5% FBS, P/S as for Vero cells and 1% tryptose phosphate broth (TPB) (Sigma-Aldrich, Saint Louis, MO).

DENV-5 strain (DKE-121), two human DENV-4 strains (INH6412 and LF32) and two sylvatic DENV-4 strains (P75-514 and P73-1120) were propagated in C6/36 cultures to obtain high titer stocks. Cell supernatants were clarified from cellular debris by low-spin centrifugation (630 x g, 10 min at 4°C), stabilized with the addition of 1X SPG (2.18 M Sucrose, 0.038M KH_2PO_4, 0.072M K_2HPO_4 and 0.054M L-glutamate), aliquoted and stored at -80°C. Viruses used in the blood meals were further concentrated through AMICON filters (membrane with NMWL of 100 kDa, Millipore) following the manufacturer's protocol and purified on a sucrose gradient. Viral titers were determined by focus-forming assay (FFA) as previously described (Vasilakis, Shell et al. 2007).

Mosquitoes

Ae. albopictus mosquitoes (Galveston colony - University of Texas Medical Branch) and *Ae. aegypti* (NIH colony and F4 generation from field collected Las Cruces NM mosquito population – New Mexico State University) were maintained at 28°C with

80% relative humidity and a 12/12 (light/dark) hour photoperiod. Mosquitoes were starved overnight prior the bloadmeal feeding.

Monkeys

Six young adult male rhesus macaques (*Macaca mulatta*) (Chinese genetic background) were inoculated subcutaneously with a dose of $1x10^3$ focus-forming units (FFU) (n=3) or $1x10^5$ FFU (n=3) of DENV-5 DKE-121 (Mayer et al., in preparation). All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) with oversight of staff veterinarians at the AAALAC-approved Animal Resources Center (ARC) at the University of Texas Medical Branch (UTMB).

Vector competence of *Ae. aegypti* and *Ae. albopictus* fed on an artificial blood meal

Artificial blood meals containing approximately 10^7 FFU/ml (see Tables 1, 2 and 3 for stock titers of viruses used) of DENV-5 DKE-121, two strains of human DENV-4 (INH6412 and LF32) and two strains of sylvatic DENV-4 (P75-514 and P73-1120) were offered in Hematek membrane feeders (Discovery Workshops, Accrington, UK) to feed 50 *Ae. aegypti* and 100 *Ae. albopictus* mosquitoes using a 1:1 mixture of virus and medium consisting of 2% (w/v) sucrose, 20% (v/v) FBS, 5 mmol of ATP, 33% (v/v) PBS-washed human blood cells (UTMB blood bank), and 37% (v/v) DMEM. Additionally two serial tenfold dilutions of DENV-5 DKE-121 were also offered in the feeders to try to define the oral infectious dose 50 (OID_{50}) of this virus. Fully engorged mosquitoes were selected and maintained for an extrinsic incubation period (EIP) of 14 days at environmental conditions of 28°C and 80% humidity. In each experiment, a small aliquot of the feeding suspension was held at room temperature for the duration of mosquito feeding, which usually continued for 1 to 2 hours. The suspension was

subsequently titrated to determine virus concentration.

Focus forming immunoassay (FIA) and immunostaining (IHC)

To assess infection and dissemination, body and legs were dissected and individually homogenized in 0.5 ml DMEM (GIBCO) supplemented with 2% FBS and 2.5 µg/ml amphotericin B (GIBCO) using a TissueLyser (Qiagen, Valencia, CA). Each homogenate was inoculated in C6/36 cell monolayers in 96-well plates (for *Ae. albopictus*) or 6-well plates (for *Ae. aegypti*), and incubated at 28°C and 5% CO_2 for 4 days. Subsequently, plates were fixed with the addition of ice-cold acetone and methanol (1:1) for 30 min at room temperature (RT). The fixation solution was aspirated and plates were allowed to air dry. Plates were then washed with PBS, followed by 30 min incubation with blocking solution (PBS supplemented with 3% FBS) and subsequent addition of mouse anti-DENV-4 or -DENV-5 ascites fluid (1:2,000) and incubation overnight at RT. The antibody was aspirated and plates washed 3 times in PBS followed by addition of secondary antibody conjugated to horseradish peroxidase (HRP) (KPL, Gaithersburg) (1:1,000) and incubation at RT for 1 hour. Plates were washed 3 times with PBS and aminoethylcarbazole (AEC) substrate (ENZO Diagnostics, Farmingdale), prepared according to the manufacturer's instructions, was added and allowed to incubate in the dark for 10 min. Substrate solution was aspirated, washed with water and plates were allowed to air dry before scoring.

Vector competence of *Ae. albopictus* fed on viremic NHPs infected with DENV-5

Fifty uninfected, 4-day old female *Ae. albopictus* were deprived of sugar water for 20-30 hours. Mosquitoes were allowed to feed in six experimentally DENV-5-infected NHPs at days 2, 3, 5 and 6 post-infection (Mayer et al., in preparation) for 5-10 min until

they became fully engorged. Fully engorged mosquitoes were selected and maintained for an extrinsic incubation period (EIP) of 14 days at environmental conditions of 28°C and 80% humidity. After 14 days, mosquitoes were dissected in body and legs, homogenized and frozen at -80°C until further analysis for the presence of the virus as described above.

RESULTS

Infectivity and dissemination levels in *Ae. aegypti* and *Ae. albopictus* fed on an artificial blood meal

Infection and dissemination of the two human-endemic and two sylvatic strains of DENV-4 as well as DENV-5 (DKE-121) in NIH colony *Ae. aegypti* are shown in Table 4.1. We detected DENV-5 DKE-121 in mosquitoes that engorged on bloodmeals spiked with 7.8 and 6.8 log10 pfu/ml of virus (infectivity of 27% and 7%, respectively) but not 5.8 log10 pfu/ml. The infectivity level by LF32 was 16%, followed by P73-1120 (9%), INH6412 and P75-514 (5%). The highest percentage of dissemination was observed for sylvatic strain of DENV-5 (DKE-121) (100%), which had an infectious dose of 6.8 logs, followed by the dose of 7.8 logs of DKE-121 (67%), P73-1120 (50%) and LF32 (33%). The strains INH6412 and P75-514 were unable to disseminate in the *Ae. aegypti* vector.

Table 4.1. Infection and dissemination of select human and sylvatic DENV-4 strains in *Ae. aegypti* (NIH colony strain)

Virus isolate (H/S)[1]	Location/ Year of Isolation	Stock Virus Titer in C6/36 cells (log_{10} pfu/mL)	No. Infected/ No. engorged	% infected	No. disseminated/ No. engorged	% absolute dissemination	No. Disseminated/ No. infected	% disseminated from infected head
INH6412 (H)	Venezuela/1985	7.5	1/21	5	0/21	0	0/1	0
LF32 (H)	Malaysia/1999	7.5	3/19	16	1/19	5	1/3	33
P75-514 (S)	Malaysia/1975	7.3	1/20	5	0/20	0	0/1	0
P73-1120 (S)	Malaysia/1973	7.5	2/23	9	1/23	4	1/2	50
DKE-121 (S)	Malaysia/2009	7.8	6/22	27	4/22	18	4/6	67
DKE-121 (S)	Malaysia/2009	6.8	1/15	7	1/15	7	1/1	100
DKE-121 (S)	Malaysia/2009	5.8	0/21	0	--	--	--	--

[1] H – DENV isolate from the human transmission cycle; S – DENV isolate from the sylvatic transmission cycle

Many previous studies have shown that a given strain of *Ae. aegypti* differs in its vector competence for different strains of DENV (Armstrong and Rico-Hesse 2003; Anderson and Rico-Hesse 2006). To extend the analysis of *Ae. aegypti*, infection and dissemination of the same five viruses was also tested in a different strain of the mosquito, the F4 generation of *Ae. aegypti* that were collected from the field in Las Cruces, New Mexico. Las Cruces *Ae. aegypti* were refractory to all the DENV-4 strains tested (Table 4.2), but susceptible to DENV-5 DKE-121 at the infectious dose of 6.8 logs, with infectivity and dissemination levels of 7% and 33%, respectively. Thus, it was demonstrated that the long-colonized NIH strain of *Ae. aegypti* was more susceptible to all strains of DENV tested than the *Ae. aegypti* strain derived from Las Cruces. This is consistent with previous findings showing that the Las Cruces strain of *Ae. aegypti* is less susceptible to DENV-4 than the NIH colony strain (Johnson et al., unpublished data).

Table 4.2. Infection and dissemination of select human and sylvatic DENV-4 strains in *Ae. aegypti* (F4 from field collected **Las Cruces NM population)**

Virus isolate (H/S)[1]	Location/ Year of Isolation	Stock Virus Titer in C6/36 cells (log_{10} pfu/mL)	No. Infected/ No. engorged	% infected	No. disseminated/ No. engorged	% absolute dissemination	No. Disseminated/ No. infected	% disseminated from infected head
INH6412 (H)	Venezuela/1985	7.5	0/23	--	--	--	--	--
LF32 (H)	Malaysia/1999	7.5	0/20	--	--	--	--	--
P75-514 (S)	Malaysia/1975	7.3	0/21	--	--	--	--	--
P73-1120 (S)	Malaysia/1973	7.5	0/19	--	--	--	--	--
DKE-121 (S)	Malaysia/2009	7.8	0/24	--	--	--	--	--
DKE-121 (S)	Malaysia/2009	6.8	3/41	7	1/41	2	1/3	33
DKE-121 (S)	Malaysia/2009	5.8	0/15	--	--	--	--	--

[1] H – DENV isolate from the human transmission cycle; S – DENV isolate from the sylvatic transmission cycle

Infection and dissemination of the two human-endemic and two sylvatic strains of DENV-4 as well as DENV-5 (DKE-121) in *Ae. albopictus* (Galveston colony) are shown in Table 4.3. Human INH6412 DENV-4 strain had infectivity level of 67.7%, followed by P75-514 (52.8%), LF32 (47.3%), P73-1120 (30.4%) and DKE-121 (12.1%). The highest percentage of dissemination was observed for sylvatic strain of DENV-4 P75-514 (32.1%), followed by LF32 (27.7%), DKE-121 (20%), INH6412 and P73-1120 (14.2%), but there were no significant differences among the DENV-4 and DENV-5 strains for this vector. Furthermore, comparison of different doses of DENV-5 revealed that *Ae. albopictus* mosquitoes were only infected with the highest dose used in the blood meal.

Table 4.3. Infection and dissemination of select human and sylvatic DENV-4 strains in *Ae. albopictus* (**Galveston colony**)

Virus isolate (H/S)[1]	Location/ Year of Isolation	Stock Virus Titer in C6/36 cells (log_{10} pfu/mL)	No. Infected/ No. engorged	% infected	No. disseminated/ No. engorged	% absolute dissemination	No. Disseminated/ No. infected	% disseminated from infected head
INH6412 (H)	Venezuela/1985	7.0	21/31	67.7	3/31	9.6	3/21	14.2
LF32 (H)	Malaysia/1999	7.0	18/38	47.3	5/38	13.1	5/18	27.7
P75-514 (S)	Malaysia/1975	7.0	28/53	52.8	9/53	16.9	9/28	32.1
P73-1120 (S)	Malaysia/1973	7.0	7/23	30.4	1/23	4.3	1/7	14.2
DKE-121 (S)	Malaysia/2009	7.0	5/41	12.1	1/41	2.4	1/5	20
DKE-121 (S)	Malaysia/2009	6.0	0/29	0	--	--	--	--
DKE-121 (S)	Malaysia/2009	5.0	0/26	0	--	--	--	--

[1] H – DENV isolate from the human transmission cycle; S – DENV isolate from the sylvatic transmission cycle

Natural infection of *Ae. albopictus* on viremic NHP

To determine whether *Ae. albopictus* (UTMB, Galveston colony) showed a different pattern in susceptibility to DENV-5 when infected via the natural route, fifty starving mosquitoes were fed on one of six DENV-5-infected monkeys at days 2, 3, 5 and 6 post-infection (pi). These days were chosen because they represent the period that viremia was detected in the DENV-5 infected NHP (see Fig.3.2A on chapter 3). One of the 6 monkeys used in this experiment transmitted DENV-5 to the mosquitoes at day 5 pi, with 4% and 100% of mosquitoes showing infection and dissemination from infected bodies, respectively (Table 4.4). Regardless of the low rate of infectivity, it was demonstrated that DENV-5 can be transmitted to mosquitoes through the NHPs. Considering that total amount of virus and duration of viremia could be important factors that reflect in vector competence of a specific mosquito strain, and in this experiment two different infectious doses ($1x10^3$ and $1x10^5$ pfu/NHP) were used, it can be speculated that the longer viremia period presented by NHP infected with low dose virus facilitated DENV-5 infectivity to *Ae. albopictus*.

Table 4.4. Infection and dissemination of DENV-5 DKE-121 strain in *Ae. albopictus* fed on viremic rhesus macaques

	Day 2					Day 3				
Animal ID	Monkey viremia (log10pfu/ml serum)	No. Infected/ No. engorged	% infected	No. Disseminated/ No. infected	% disseminated	Monkey viremia (log10pfu/ml serum)	No. Infected/ No. engorged	% infected	No. Disseminated/ No. infected	% disseminated
98091[a]	≤1.0	0/10	0	0	0	≤1.0	0/33	0	0	0
88249[a]	≤1.0	0/12	0	0	0	2.3	0/21	0	0	0
98041[b]	2.3	0/20	0	0	0	2.9	0/7	0	0	0
98029[b]	2.7	0/29	0	0	0	2.8	0/33	0	0	0
76165[b]	2.8	0/20	0	0	0	3.3	0/25	0	0	0
76135[a]	≤1.0	NT	0	0	0	≤1.0	0/8	0	0	0
	Day 5					Day 6				
98091[a]	2.9	0/4	0	0	0	3.1	0/10	0	0	0
88249[a]	3.7	1/27	4%	1/1	100%	3.6	0/19	0	0	0
98041[b]	≤1.0	0/23	0	0	0	≤1.0	0/19	0	0	0
98029[b]	≤1.0	0/20	0	0	0	≤1.0	0/20	0	0	0
76165[b]	3.3	0/24	0	0	0	1.6	0/16	0	0	0
76135[a]	3.3	0/16	0	0	0	3.7	0/20	0	0	0

a. Monkey inoculated with 3.0 log10pfu
b. Monkey inoculated with 5.0 log10pfu

DISCUSSION

Vector competence for arboviruses is greatly affected by the extensive differences within mosquito vector species. Several studies based on the mosquito genetics showed specific molecular markers closely related to the genetic differentiation present among mosquito populations and also the existence of a general genetic difference among mosquito species (Cui, Qiao et al. 2007; Huang, Molaei et al. 2008; Edillo, Kiszewski et al. 2009; Fonseca, Smith et al. 2009; Sharma, Mendki et al. 2009; Weitzel, Collado et al. 2009). In the present study it has been demonstrated that the newly emerged DENV-5 showed similar levels of infectivity when compared to two human strains and two sylvatic strains of DENV-4 in *Ae. aegypti* vector. Similar dissemination rate was also observed in this vector. However, when the same DENV strains were used to determine the vector competence in *Ae. albopictus*, it was observed that the highest levels of infectivity were for the human DENV-4 strain INH6412 and the lowest for DENV-5. Nevertheless, dissemination level in *Ae. albopictus* vector did not showed variation among DENV strains. When comparing the infectivity level between *Ae. aegypti* and *Ae. albopictus*, it was observed a higher infectivity in *Ae. albopictus* vector. However, dissemination levels were higher in *Ae. aegypti* for all the viruses tested included DENV-5. These *Ae. aegypti* infectivity data are in agreement with Gubler and his group where they observed variation in susceptibility of different strains of *Ae. aegypti*, but the susceptibility patterns were similar among different DENV serotypes (Gubler, Nalim et al. 1979). However, other studies of DENV infection rate in the field-caught population of female *Ae. aegypti* and *Ae. albopictus* mosquitoes in Singapore demonstrated a higher infection level in *Ae. aegypti* compared to *Ae. albopictus* vector from the period of 1997

to 2000 (Chung and Pang 2002). Also, it was observed DENV-1 as being the most prevalent serotype (Chung and Pang 2002). DENV infectivity studies using several strains of *Ae. albopictus* demonstrated a significant variation in susceptibility for each of the four DENV serotypes among vectors collected in different geographic regions (Gubler and Rosen 1976). Another study evaluated the vector competence of *Ae. aegypti* in the Island of Santiago, Cape Verde for the four DENV serotypes and demonstrated that the local mosquito population had a high vector competence for DENV serotypes 2 and 3 and a low vector susceptibility for DENV serotypes 1 and 4 (da Moura, de Melo Santos et al. 2015). The same study also observed that DENV-4 had lower replication level compared to the other three DENV serotypes, suggesting that the population of *Ae. aegypti* from Cape Verde has an efficient midgut escape barrier (MEB) against DENV-4, which could reflects in the capability of the virus to disseminate to the salivary glands (da Moura, de Melo Santos et al. 2015). Studies using two strains of DENV-2 serotype (DEN-2 43 and NGC) have showed that *Ae. albopictus* midgut and salivary glands are infected with both virus strains, however DENV-2 NGC strain was able to generate greater percentage of midgut infections than DENV-2 43 strain (Guo, Zhu et al. 2013). These data indicate that the higher dissemination level observed for NGC strain could be the result of an increased viral replication of DENV-2 NGC in *Ae. albopictus*. However, when the authors investigated the salivary gland infection rate, no difference was observed between both DENV-2 strains (Guo, Zhu et al. 2013). In addition, it was demonstrated that *Ae. aegypti* was more susceptible to infection by NGC strain than DENV-2 43 (Guo, Zhu et al. 2013). Other studies of vector competence in *Ae. aegypti* mosquitoes to DENV-2 in two cities of Kenya (Nairobi and Kilifi) concluded that the

vector population from Nairobi was an inefficient vector for DENV-2 when compared to the vector population from Kilifi. The *Ae. aegypti* population from Nairobi had a weak midgut infectious barrier (MIB) and a strong MEB, in contrast to *Ae. aegypti* from Kilifi that showed a moderate MIB and a weak MEB (Chepkorir, Lutomiah et al. 2014). Vector competence studies have been investigated comparing DENV serotypes 2 and 4 for *Ae. aegypti* in four different regions of Australia (Torres Strait, Charters Towers, Townsville, and Cairns). Mosquito populations from Torres Strait were described to be more susceptible to DENV-2 and were more efficient in viral transmission than mosquito from the other three regions. When vector competence was determined for DENV-4 serotype the group also demonstrated higher levels of susceptibility in mosquito from Torres Strait region, however there was not significant difference in the transmission levels among the regions for this serotype (Knox, Kay et al. 2003). Vector competence disparities have been extensively described among DENV isolates and different *Ae. aegypti* strains and it can be directly correlated to genetic alterations (Gubler, Nalim et al. 1979; Tardieux, Poupel et al. 1990; Tran, Vazeille-Falcoz et al. 1999; Vazeille-Falcoz, Mousson et al. 1999; Bennett, Olson et al. 2002; Mousson, Vazeille et al. 2002). Mutually, several studies have also showed that a certain mosquito strain present variability in infectivity for some DENV isolates (Armstrong and Rico-Hesse 2003; Anderson and Rico-Hesse 2006).

The present study evaluated vector competence of *Ae. aegypti* and *Ae. albopictus* to a newly emerged DENV serotype and compared to sylvatic and human DENV-4 strains. Similar infectivity and dissemination among DENV-5 and DENV-4 strains were observed. Additionally, DENV-5 DKE-121 isolate was able to be disseminated in a field-

caught mosquito population (Las Cruces) showed to be less susceptible to other DENV-4 strains. Many studies have documented differences among populations of *Ae. aegypti* in vector competence for DENV (Gubler, Nalim et al. 1979; Tardieux, Poupel et al. 1990; Tran, Vazeille-Falcoz et al. 1999; Vazeille-Falcoz, Mousson et al. 1999; Bennett, Olson et al. 2002; Mousson, Vazeille et al. 2002). Here it is notable, and rather worrying, that the only infection observed in the low-susceptibility Las Cruces *Ae. aegypti* strain was by DENV-5 DKE-121. More importantly, it was demonstrated the infection of *Ae. albopictus* mosquitoes feeding on viremic NHPs infected with DKE-121, suggesting this newly emerged DENV serotype can establish a sylvatic transmission cycle with the potential of spillover to the human population.

CHAPTER 5: Cryo-Electron Microscopy (Cryo-EM) Reconstruction of Dengue Virus Serotype 5 (DENV-5, DKE-121)

INTRODUCTION

Dengue virus (DENV) belongs to the *Flavivirus* genus into the *Flaviviridae* family and has a positively charged RNA genome of a size of 10.5 Kb. The immature and mature particles have a diameter of approximately 600Å and 500Å, respectively. The glycoprotein shell is composed of envelope (E) and membrane (M) proteins surrounded in a lipid bilayer derived from the cell host. The viral genome inside the virus particle is aggregated with the capsid protein (Kuhn, Zhang et al. 2002). It has been demonstrated that the surface of DENV particles present a highly dynamic characteristic as the virion can endure major structural changes at different stages of the infection cycle (Mukhopadhyay, Kuhn et al. 2005). One of the first flavivirus high-resolution studies was achieved by Rey and collaborators, where they demonstrated for the first time the envelope glycoprotein from tick-borne encephalitis virus (TBEV) at 2Å resolution (Rey, Heinz et al. 1995). DENV low-resolution studies were performed by Kuhn and collaborators, where they were able to reconstruct the virus particles at 24Å using cryo-EM and observed that the virus assumed icosahedral symmetry (Kuhn, Zhang et al. 2002). The structural reconstruction were found to be in agreement with previous flavivirus studies (Burke and Monath 2001; Lindenbach, Pragai et al. 2007). Other DENV studies showed better reconstruction at 9.5Å, showing a secondary structure disposition of 180 copies of the E and 180 copies of the M proteins in the lipid envelope. This investigation made possible the observation that the α-helical stem regions of the E

protein molecules, as well as part of the terminal section of the M protein were buried in the outer leaflet of the viral membrane (Zhang, Hunke et al. 2012). However only high resolution studies (3.5 Å) in DENV mature particles were able to shown the latch-type interaction between the E and M proteins and determine that this interaction was mediated by pH sensitive residues that embraces E in place and blocks premature exposure of its fusion peptide (Zhang, Hunke et al. 2012). Moreover, a recent DENV study using high-resolution cryo-EM reconstruction compared DENV-4 with DENV-1 and DENV-2 serotypes. This study found differences in charge distribution among the serotypes that could explain the differences in the virus binding to cellular receptors (Kostyuchenko, Chew et al. 2014). Other important observation described in the study was the variation in amino acid residues involved in the interaction between the E and M proteins (Kostyuchenko, Chew et al. 2014). Moreover, the comparison of DENV-4 structures demonstrated the occurrence of differences in the virus shell characteristics among the other DENV serotypes (Kostyuchenko, Chew et al. 2014). Near atomic resolution studies are extremely relevant as they enable to locate regions that are crucial in conferring structural stability and also to collect information that is important to investigate virus interaction with cellular receptors, antibodies and drugs. The objective of the current study was to determine the high-resolution reconstruction of DENV-5 to compare differences in the virus structure to the other DENV serotypes.

MATERIALS AND METHODS

Cells

Vero cells were maintained at 37°C in Dulbecco's Minimal Essential Medium (DMEM) (GIBCO, Life Technologies, Carlsbad, CA) supplemented with 5% heat inactivated fetal bovine serum (FBS) (Hyclone, Thermo Scientific, Logan, UT) and 100U/mL of penicillin and 100μg/mL of streptomycin (P/S) (GIBCO). C6/36 cells were propagated at 28°C in DMEM supplemented with 5% FBS, P/S as for Vero cells and tryptose phosphate broth (TPB) (Sigma-Aldrich, Saint Louis, MO).

Viruses

Low passage DENV-5 isolate (DKE-121 strain) was propagated in C6/36 cultures to obtain high titer stocks. Cell supernatants were clarified from cellular debris by low-spin centrifugation (630 x g, 10 min at 4°C), stabilized with the addition of 1X SPG (2.18 M Sucrose, 0.038M KH2PO4, 0.072M K2HPO4 and 0.054M L-glutamate), aliquot and stored at -80°C. Viral titers were determined by focus-forming assay (FFA) as previously described (Vasilakis, Shell et al. 2007).

Virus preparation for Cryo-EM analysis

Virus was grown in large scale using 20 to 30 T-150 flasks of C6/36 cells at confluence of 80% at the time of infection. Cells were infected at the multiplicity of infection (MOI) of 0.1 and incubated for 7 days at 28°C and 5% CO_2 atmosphere. At day 7 post-infection (pi), the cell supernatant was harvested and centrifuged at 4,000 rpm at 4°C for 10 minutes with the objective to remove cell debris. After centrifugation, supernatant was collected and filtered using 0.22 μm filter units (Thermo Scientific). Virus was further concentrated through AMICON filters (membrane with NMWL of 100 kDa, Millipore) following the manufacturer's protocol. The concentrated virus was further purified through potassium tartrate-glycerol gradient ultracentrifugation for 18

hours as previously described (CR Ashley and E O Caul 1982). After ultracentrifugation, the fraction containing the virus was washed with 1X TEN buffer and sent immediately to UTMB core lab for cryo-EM study. A small aliquot of the virus was kept to quantify the viral titer by focus-forming assay (FFA) as previously described (Vasilakis, Shell et al. 2007).

Cryo-EM microscopy and image data processing

The manipulations with DENV-5, including sample vitrification, grid transfers and microscopy were performed at the UTMB cryo-EM core lab using the appropriated personal protection equipment (PPE). All the procedures for cryo-EM reconstruction of DENV-5 were followed as previously described (Sherman, Guenther et al. 2006; Freiberg, Sherman et al. 2008; Sherman and Weaver 2010). Briefly, virus particles were vitrified on holey carbon film grids and the frozen grids were transferred to a cryo-specimen 626 holder before been loaded to the electron microscope. Grids were kept at liquid nitrogen temperature (-172 to -180°C) during imaging acquisition. Viruses were imaged with a nominal magnification of 60,000. In total, 4,500 individual virus images were collected and selected images were used in the final DENV-5 reconstruction.

RESULTS AND DISCUSSION

The advantages offered by cryo-electron microscopy (cryo-EM) are abundant in the determination of structures of biological machines. Samples submitted to cryo-EM studies are analyzed in numerous forms and shapes, such as two or three-dimensional crystals of single particles with or without symmetry (Saibil 2000; Gao, Sengupta et al. 2003; Jiang, Li et al. 2003). Considering the chances of structural elements to be present

different from crystalized forms, analyzes using cryo-EM are able to predict the machine structure in its native interacting states that are significant to cellular functions (Chiu, Baker et al. 2005). In this study it is described the first cryo-EM reconstruction of a newly emerged DENV-5 serotype. The diameter of the mature DENV-5 particle was approximately 500 Å and the resolution achieved for the cryo-EM map was 18Å (Fig.5.1). Cryo-EM images showed spherical particles (Fig.5.1A), which is the typical morphology of DENV. The density map of cryo-EM reconstruction of the mature DENV-5 particles showed a relative smooth surface (Fig.5.1B). A central cross-section revealed a traditional structure of DENV, showing the nucleocapsid surrounded by E glycoprotein (Fig.5.1C).

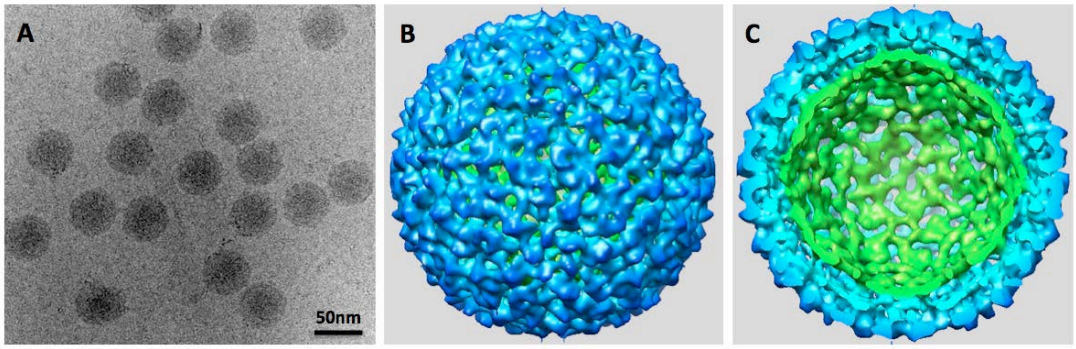

Figure 5.1: **Structure of DENV-5 DKE-121.** (A) Cryo-EM image showing virus particles embedded in vitreous water. Scale bar represents 50 nm. (B) Three-dimensional reconstruction of DENV-5 particles at 18Å resolution. (C) Central cross-section showing the density maps for nucleocapsid (green) and E glycoprotein (blue).

Although the reconstruction of different DENV serotypes is similar in several aspects, there are differences among DENV serotypes that can affect the virus function *in vitro* and *in vivo*. Reconstruction at high resolution of all serotypes is extremely relevant to the development of safe vaccines and effective therapeutics as well as studies of

infectivity (Kostyuchenko, Zhang et al. 2013; Zhang, Ge et al. 2013). Recent studies compared differences in heparan sulfate dependence of DENV-2 and other DENV serotypes (Lin, Lei et al. 2002). Their research demonstrated that the infectivity of DENV-2 was highly dependent on heparan sulfate (HS), and through cryo-EM analyses they found that DENV-2 had more positively charged residues in the E protein when compared to the other serotypes. These observation could explain the great preference of DENV-2 for cells expressing higher concentrations of HS, mostly because this molecule has affinity for positively charged residues (Lin, Lei et al. 2002).

High resolution studies of DENV-4 has shown that although the general organization of shell proteins in this serotype being very comparable to the other serotypes already described in cryo-EM studies, there are characteristics in the surface of the virus that are clearly distinct (Kostyuchenko, Chew et al. 2014). The study also explored differences in the amount of connections that sustain the shell proteins among different serotypes, which could explain the differences in the capability to experience structural modification at elevated temperatures (Kostyuchenko, Chew et al. 2014).

Based on the current knowledge about DENV structure, further investigation of the structure of DENV-5 at near-atomic resolution will be important to determine differences among the other DENV serotypes, including mechanisms of virus neutralization, receptors binding, infectivity and many other characteristics. These will not only affect the basic knowledge of the structure of the newly emerged DENV serotype, but also will help in the design of therapeutic drugs and vaccines if the virus establish a continuous transmission cycle among human beings.

CHAPTER 6: Production of Monoclonal Antibodies using Cells Derived from Non-Human Primates Inoculated with Dengue Virus Serotype 5 (DENV-5, DKE-121)

INTRODUCTION

The development of monoclonal antibodies (MAbs) was made possible by studies from Georges J.F.Kohler and Cesar Milstein in 1975. The hallmark in this area was the development of hybrids between mouse splenocytes and a myeloma cell partner to generate a specific antibody-producing cell (Kohler and Milstein 1976). A few years later the scientists were recognized for their outstanding contribution and received the 1984 Nobel Prize in Physiology or Medicine shared with Niels K. Jerne. Since that time significant advances have been made, changing the face of biomedicine and the contribution of monoclonal antibodies in clinical application (Alkan 2004; Geskin 2015).

Antibodies are proteins produced by the host, which are found in the plasma and extracellular fluids. These proteins function as one of the first specific immune response and are considered one of the fundamental effector molecules of the adaptive immune system. The host produces antibodies in reaction to pathogens and other antigens in the attempt to promote neutralization or elimination of the foreign organism or molecule. Antibodies have the capability to attach to the antigen with high level of affinity and specificity, which contribute their universal use in a diversity of scientific and medical applications. They had an insightful impact on the progress of human health as well in animal welfare due their extensive use as diagnostic tool and therapeutic application (Lipman, Jackson et al. 2005).

Non-human primates (NHPs) play a relevant role in elucidation of several aspects of human diseases, mostly because they have a close genetic relationship with humans (Wang, Niu et al. 2012). Previous studies of B cell characterization in cynomolgus monkeys (*Macaca fascicularis*) have described that B cell subsets from this specie have some characteristic being very similar to those present in other NHP species as well as those present in humans. The study had demonstrated that the number of B cells presenting a memory phenotype are similar in the blood stream of human and cynomolgus monkeys (Vugmeyster, Howell et al. 2004). Resemblance of NHP and human antibodies is the subject of several studies. Some studies described that immunoglobulin (Ig) genes of NHP are closest to human genes then among different NHP species themselves (Andris-Widhopf, Steinberger et al. 2001; Pelat and Thullier 2009). Another study demonstrated that the immunoglobulin variable region genes from NHPs have shown to have 85-98% homology with human immunoglobulin sequences (Newman, Alberts et al. 1992). For many different reasons, studies employing immunization of NHP is more reasonable then using human subjects. Variable (V) domains source of NHPs are suitable to use with constant regions of human IgGs and facilitate the generation of primatized antibodies. Examples of primatized antibodies already in clinical trials are the anti-CD80 (anti-B7), IDEC-114 used for psoriasis (Schopf 2001) and an anti-CD23 Ab, IDEC-152 (lumiliximab) used for allergic asthmatics patients (Rosenwasser, Busse et al. 2003). In addition, NHPs have played a crucial role in dengue virus (DENV) studies concerning comparison of viremia between different serotypes, antibody kinetics during immune response for vaccine evaluation as well as therapeutic evaluation (Halstead, Shotwell et al. 1973; Koraka, Benton et al.

2007; Koraka, Benton et al. 2007).

The aim of this study was to establish the development of monoclonal antibodies generated from NHP immunized with DENV-5 DKE-121. NHP cells from blood, bone marrow and spleens were used in the fusion procedure with the myeloma cell line to obtain hybridomas that potentially secreted DENV-specific monoclonal antibodies.

MATERIALS AND METHODS

Virus propagation

Low passage DENV-5 isolate DKE121 was propagated in C6/36 cell cultures to obtain high titer stocks. Cell supernatants were clarified from cellular debris by low-spin centrifugation (630 x g, 10 min at 4°C), stabilized with the addition of 1X SPG (2.18 M Sucrose, 0.038M KH2PO4, 0.072M K2HPO4 and 0.054M L-glutamate) and aliquots were stored at -80°C. Viral titers were determined by focus-forming assay (FFA) as previously described (Vasilakis, Shell et al. 2007).

Animals

Young adult male rhesus macaques (*Macaca mulatta*) tested seronegative for flavivirus infections were used in this study (see Chapter 3 for more details). One group of animals (n=3) was inoculated subcutaneously (s.c.) with $1x10^3$ focus-forming units (FFU) and the second group (n=3) with a dose of $1x10^5$ FFU of DENV-5 DKE-121. All animal work was approved by the Institutional Animal Care and Use Committee (IACUC) with oversight of staff veterinarians at the AAALAC-approved Animal Resources Center (ARC) at the University of Texas Medical Branch (UTMB).

Tissue collection and single-cell preparation

Whole blood was collected from NHPs inoculated with DENV-5 two months after homotypic challenge. The separation of buffy coat to obtain peripheral blood mononuclear cells (PBMCs) was performed through gradient centrifugation using histopaque-1083 (Sigma-Aldrich), according manufacturer protocol. After separation the cells were counted and used for the fusion procedure (described below). In the initial experiments, B cells were sorted from PBMCs using non-human primate CD20 MicroBeads (Miltenyi Biotec) following the manufacturer protocol.

Spleens were collected under sterile conditions and placed into a container on ice at the end time point of the experiment (day 350 pi). To make single-cell suspension, each spleen was placed into a Petri dish containing RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) (Hyclone), the connective tissue and fat were removed and the spleen was cut in small pieces. Cells were individualized by passing through a cell mesh screen (Sigma-Aldrich). Cells were washed by centrifugation, counted and part was used for fusion procedure and part was cryopreserved in 10% dimethyl sulfoxide (DMSO) and 90% FBS for future use. Bone marrow cells were also extracted from the femurs of the NHPs. A longitudinal section was made in both extremities of the bone (epiphysis and mataphysis) containing the red marrow (spongy bone) and cells were flushed out by injecting medium using a 10 mL syringe and 18G needle. Bone marrow cells were washed, counted, and used for fusion procedure or cryopreserved as described above.

Myeloma cells

The P3X63 Ag8.653 myeloma cells (ATTC, Manassas, VA) resistant to 8-azaguanine and HAT sensitive (Kearney J et al 1979) were grown in RPMI-1640 medium

supplemented with 10% FBS. Prior to at least 3 days of the cell fusion procedure, cells were sub-cultured daily to maintain exponencial growth.

Fusion procedure

Myeloma cells at exponential growth phase were collected and counted. Different ratio of myeloma cells to PBMCs, bone marrow or splenocytes were used depending on cell type and total cell numbers. For PBMCs and bone marrow cells, a ratio of 1:3 and 1:5 were used. For splenocytes, an additional ratio (1:10) was included. PBMCs were always used fresh, while bone marrow cells and splenocytes were used fresh or taken from the frozen stock. The mixture of cells containing the appropriated ratio was washed twice with RPMI-1640 medium containing no additives. The cell pellet was loosening by finger-flicking and fusion was performed at 37°C waterbath by slow addition of 1 mL of PEG 1000 (Roche) followed by 1 min incubation period. Warm RPMI-1640 medium was then slowly added, 1 ml in the first minute interval, followed by 3 ml in the second minute and 16 ml in the third minute interval. After fusion, cells were washed twice in RPMI-1640 medium and the pellet was resuspended in Hybridoma-SFM medium (GIBCO) containing 10% FBS and hypoxanthine-aminopterin-thymidine (HAT) medium (Invitrogen). Cells were plated in 96-well plates at density of 10^5 cells/well. Cells were refed with fresh growth medium containing HAT every 2 or 3 days for the period of 3 weeks. Hybridoma clones from individual wells were expanded and the supernatant tested for the secretion of DENV-specific antibodies.

Hybridoma screening

Supernatant of hybridoma clones were tested initially by ELISA, using DENV-5 DKE-121 as antigen. Each clone presenting a positive or suspected DENV-specific

reactivity were further expanded and cryopreserved for future single-cell cloning and additional testing. Selected clones were also tested by indirect immunofluorescence assay (IFA) or immunohistochemistry (IHC) using C6/36 cells infected with DENV-5 DKE-121.

RESULTS AND DISCUSSION

Initially, three fusions were performed using sorted B cells from PBMCs of DENV-5-infected NHPs. Although the fusion processes were successful, the clones did not survived more then a few days or a week at the most. The speculation was that likely when the fusion was performed using sorted B cells, some growth factors such as cytokines produced by other immune cell types such as macrophages and dendritic cells were not present, which could have played a role to keep the cells alive during the first days post-fusion. To overcome this problem the protocol was modified and fusion process was executed using PBMCs without sorting B cells. This strategy was successfully as the cells generated in this procedure survived after the addition of the selective medium containing HAT, indicating they were truly hybridomas (Fig.6.1). Another important aspect that was considered was the small percentage of plasma B cells in the blood. Due to this circumstance and to ensure that truly DENV-specific antibody secreting cells were produced, splenocytes and bone marrow cells from the macaques challenged with DENV-5 were used in the fusion process. Taking this approach, the probability of collecting cells from organs where plasma B cells are in abundance is enhanced, which promotes better success in obtaining plentiful numbers of DENV-

specific plasma B cells and consequently increasing the chances to generate monoclonal antibodies against DENV-5.

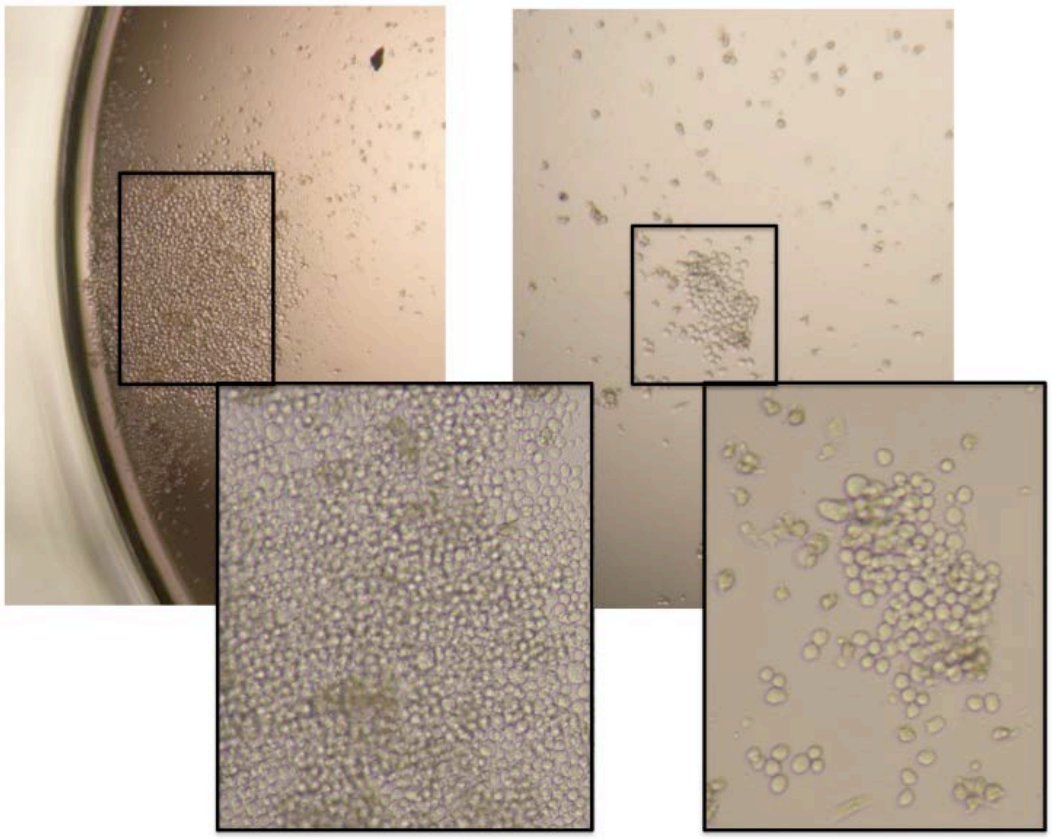

Fig. 6.1. Production of monoclonal antibodies. Micrograph showing two hybridomas after 2 weeks on selective medium at different stages of growth.

Several fusions were performed and the SOP (standard operation procedure) was established. In one particular fusion, 264 hybridomas were generated, expanded and stored appropriately. They were initially screened by ELISA. From the 264 hybridomas tested, 9 were suspect by ELISA (Fig 6.2). They were further tested by IFA and three of them presented a positive reaction (Fig 6.3). Single-cell cloning using limiting dilution was performed to select single clones secreting DENV-specific monoclonal antibodies.

Although several clones were screened, so far none of the selected clones presented a strong positive reaction by ELISA test.

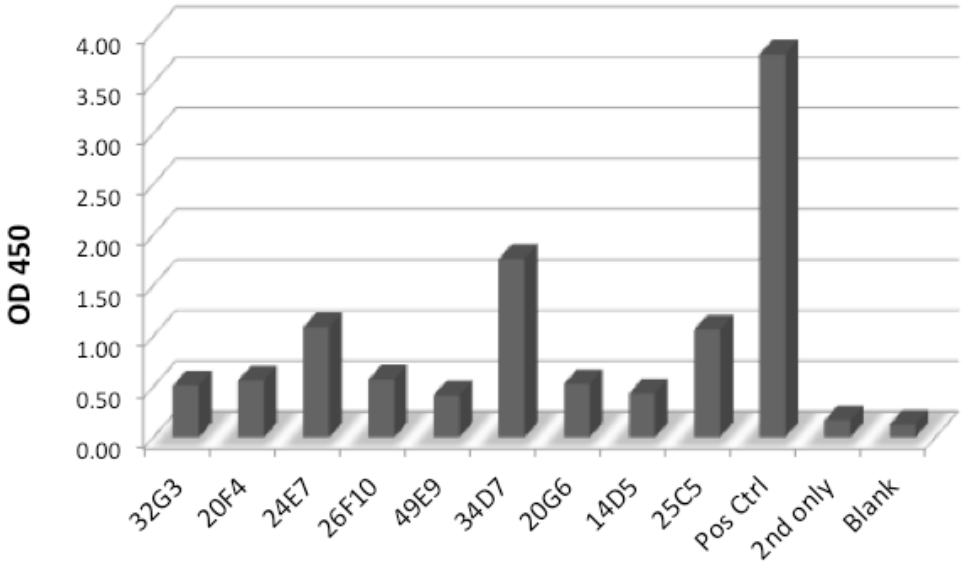

Fig. 6.2. Hybridoma screening. Cell supernatant from individual hybridoma clones were tested by ELISA.

There is much evidence that for some particular agents the production of monoclonal antibodies (MAbs) in non-human primates (NHPs) is rather advantageous when compared with the mouse model. One good example is the development of MAbs against malaria, where the parasite antigen can be presented directly from the NHP own erythrocytes (Stanley and Reese 1985). In the mouse model, a great percentage of MAbs bind to antigens of uninfected erythrocytes, while the majority of MAbs made in NHP are known to bind to the parasites or with the antigens at the parasite membrane (Stanley and Reese 1985). This is important in the elimination of intense background caused by anti-erythrocyte response that is extremely common when mouse Mabs are utilized (Stanley and Reese 1985).

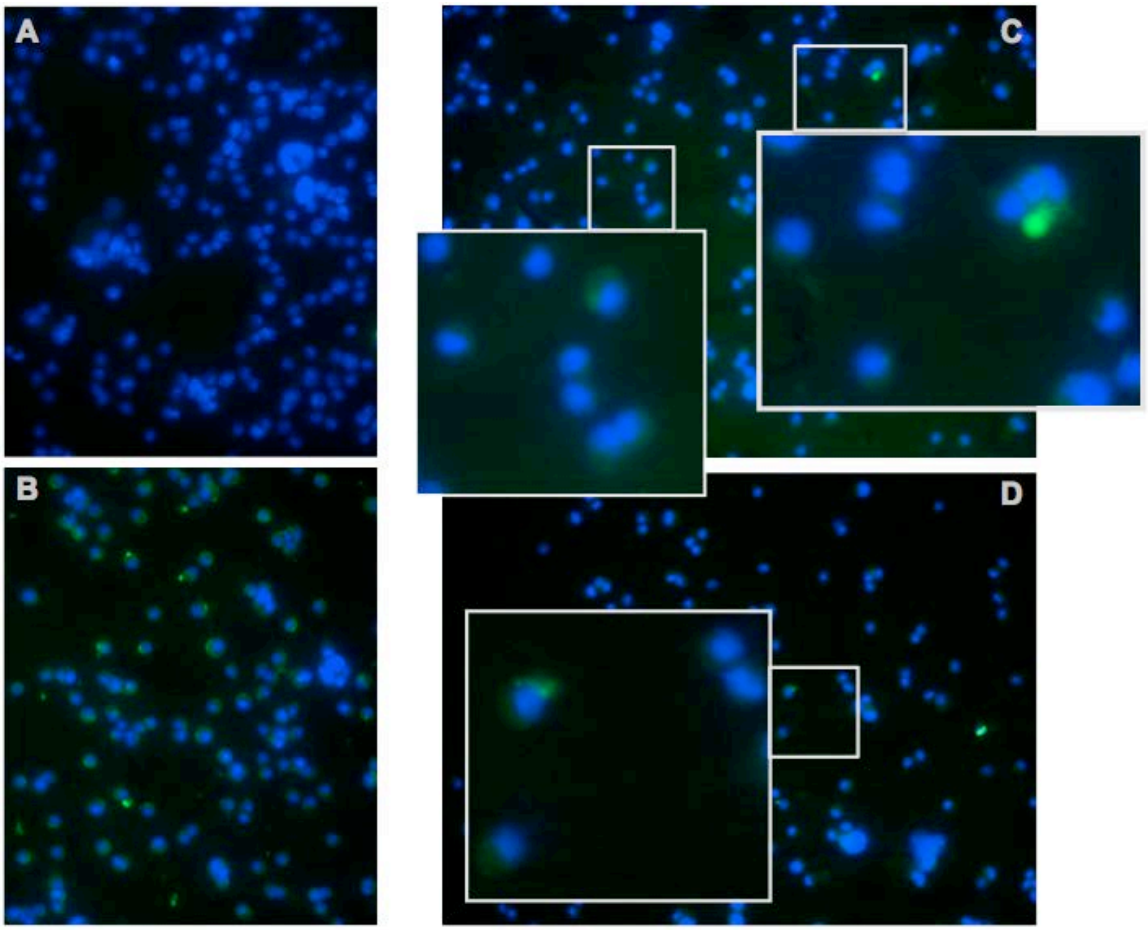

Fig. 6.3. Hybridoma screening. Cell supernatant from individual hybridoma clones presenting a suspected or positive reaction on the ELISA were tested by an indirect immunofluorescence assay (IFA) using C6/36 cells infected with DKE-121. (A) Negative control. (B) Positive control. (C) Clone 24E7. (D) Clone 20F4. Nuclei stain (DAPI) in blue and DENV E protein stain (FITC) in green.

This study described the production of stable hybridoma using NHP cells from animals exposed to the new DENV serotype. Although the hybridomas originated were stable, they secreted antibodies for only a short period of time. One possibility to explain the transient secretion of monoclonal antibody by these stable hybridomas was the use of a mouse cell line as the fusion partner instead of a chimera human-mouse cell line. Similar results were described in a previous study using a mouse monoclonal antibody

(OKT4- IgG2b) as antigen (Van Meurs and Jonker 1986). Two cell lines of myeloma were tested as fusion partners: (i) a mouse myeloma and (ii) a mouse-human heteromyeloma for the production of monoclonal antibodies using NHP lymphocytes. Similarly, the group demonstrated the transient secretion of monoclonal antibodies when they fused primate lymphocytes with the mouse myeloma; however when they used mouse-human heteromyelomas, they observed stable hybridomas cells (Van Meurs and Jonker 1986) . Another interesting outcome from the study was that only monoclonal antibodies using chimpanzee lymphocytes produced a long lasting hybridoma secreting antibody contrasting with those produced using rhesus macaques lymphocytes that stopped the antibody secretion one month after they being generated, even though mouse-human heteromyeloma has been utilized as the fusion partner (Van Meurs and Jonker 1986). One explanation for the chimpanzee's lymphocytes to be more efficient in producing stable hybridomas is due the presence of chromosomes closely related to the mouse-human heteromyeloma, considering that chimpanzees are closest related to humans then rhesus macaque. Another strategy used to increased the efficiency and the length of monoclonal antibody secretion is the cell immortalization by using Epstein-Barr virus (EBV) (Fraussen, Vrolix et al. 2010) before the fusion process. However, this alternative approach has been described to be lees efficient when using the mouse system (Van Meel, Steenbakkers et al. 1985).. Additionally, it is crucial to carefully choose the appropriate fusion partner for human and primate hybridomas to increased the success not only to obtain stable hybridomas, but also to obtain stable antibody-secreting cells. Furthermore, the addition of differentiation factors or cytokines such as interferon after fusion could improve the efficiency process of hybridoma generation.

In summary, since cell stocks including splenocytes and bone marrow cells from the DENV-5 immunized NHPs are still available for the continuation of this work, all the aspects described above should be considered to increase the probability to generate hybridomas that will sustain the production of NHP monoclonal antibodies for a longer period of time.

CHAPTER 7: Conclusions and Future Directions

Emerging infectious diseases represent a large threat to the human population. Viral diseases including illnesses caused by arboviruses affect hundreds of millions of people worldwide every year. With the advance of science, new insights in understanding the pathogenesis have become available and control measures have been developed to prevent or contain large outbreaks of several infectious diseases. Nevertheless, for many illnesses, even with years of research, an effective approach to control their occurrence still need to be investigated. Other diseases are emerging in forms that were not seen in the past and the reasons most of the times are not promptly understandable, emphasizing the importance to constantly study the origin, emergence and establishment of infectious diseases. In the past decades, arboviral epidemics have significantly increased with the exception of few regions of the world not affected by them. In most cases, the emergence of arboviral diseases were caused by viruses considered to be controlled or recognized as harmless for the public health. One of the most currently explored arbovirus in humans is DENV. Emergence of distinct DENV serotypes occurred independently and repeatedly in allopatric regions prior to their expansion in sympatric regions, using similar non-human primate hosts. Genetic studies demonstrated DENV was dispersed rapidly into new locations with the advent of air travel that enabled the movement of humans during the viremic phase of infection, resulting in the shift or extinction of local lineages. Ecological factors are also involved in the emergence of DENV. Deforestation is one of the crucial factors driving sylvatic DENV emergence. As people are exploring new resources deep into the forest, living in areas previously unexplored, the chances of sylvatic DENV

emergence are also increasing. Regions in Asia and Africa, where rapid and uncontrolled urbanization takes place, the risk of sylvatic dengue emergence is high. In this study I evaluated the following hypotheses:

The dengue virus isolated from a febrile patient in Malaysian state of Sarawak, represents the prototype virus of a new dengue serotype

This hypothesis was evaluated in chapter II. The virus isolated from a clinical case from a febrile patient from Malaysia was designated as DKE-121. After received the virus sample I amplified it in C6/36 cells to generate adequate virus stocks. The full genomic DENV sequence of DKE-121 was later obtained by *de novo* next generation sequencing (NGS). Following the virus sequencing, I stressed out the phylogenetic relationship of this isolated with the other DENV serotypes. While phylogenetic analysis of the DKE-121 strongly suggests its sylvatic origin, it is substantially divergent from its closest relative, sylvatic DENV-4. Importantly the branch length of the divergent DKE-121 is deeper than the branch length of DENV-2 emerging from its ancestral progenitors but shallower than the branch lengths of the sister serotypes DENV-1 and DENV-3, suggesting the unique origin of this virus and implying the emergence and detection of a new dengue serotype. Further, my cross-neutralization data indicated a 4-fold difference in cross-neutralization between homologous and heterologous neutralizing antibody titers. I used the 80% endpoint to estimate conservatively *in vivo* protection. However, limited cross-reactivity among some of the serotypes is observed underlying the presence of cross-neutralizing epitopes, which are thought to play a role in the observed pathogenicity of heterologous infections in nature. I subsequently evaluated the neutralization activity of a panel of well-characterized monotypic human sera against

DKE-121 virus and the other four well-established DENV serotypes. I utilized a panel of 12 monotypic DENV sera and 3 naïve sera obtained from Dr. Rick Jarman of Walter Reed Army Institute of Research (WRAIR), Viral Diseases Branch. The neutralization assays were performed using the prototypic viruses of each of the 4 DENV serotypes (DENV-1 Hawaii; DENV-2 NGC or 16681; DENV-3 H87; DENV-4, H241) and DKE-121. I have used serotype specific MIAFs for the development of the assay and an 80% endpoint to estimate conservatively *in vivo* protection. My cross-neutralization data indicate a 4-fold difference in cross-neutralization between homologous and heterologous neutralizing antibody titers. Surprisingly, DKE-121 is neutralized as equally as DENV-4 by its homotypic sera, suggesting that DKE-121 and DENV-4 viruses share antigenic epitopes with strong neutralizing potential. Based on the close genetic and evolutionary relationships of DKE-121 with DENV-4 viruses it's likely that DKE-121 shares a close antigenic relationship to DENV-4, similar to DENV-1 to its sister clade of DENV-3. My data using phylogenetic studies, cross neutralization and the antigenic cartography of human and NHP sera is suggestive that the newly emerged DKE-121 is a prototype of a sylvatic DENV-5 serotype.

The newly isolated dengue virus serotype is able to infect rhesus macaque causing viremia and stimulating a robust neutralization activity

This hypothesis was tested in chapter III. I utilized rhesus macaque (*Macaca mulatta*) as a model for two following reasons (i) NHP is the vertebrate host involved in the transmission and maintenance of DENV in the sylvatic cycle. (ii) The newly emerged DENV virus was isolated from a sylvatic cycle; so I understand by using a natural host would be the appropriate approach for this virus characterization. I utilized two genetic

backgrounds of NHP, Asian and Indian rhesus macaques to infect with DENV-5 strain. After the primary infection, I challenged one group with a homologous DENV-5 DKE-121 and the other with a heterotypic DENV-4 serotype, being one sylvatic and one human strain. My *in vivo* data using NHP as animal model demonstrated that DENV-5 was able to replicate and viremia was detected all animals infected with DENV-5. Also, in my experiment using rhesus macaques from different genetic backgrounds I was able to demonstrate that DEN-5 was able to infect and viremia was detected in one of four animals. Even though I was not able to detect viremia in all four animals in this experiment, I was able to show that all animals were successfully infected with DENV-5 based on antibodies levels, as represented through IgM levels. When I measured IgM levels after the homotypic challenge, I did not observed any reasonable increased levels of IgM, what is consistent with homotypic immune stimulation. However, after a heterotypic challenge with DENV-4 serotype, I observed a small peak of IgM levels, characterizing a typical secondary immune response, very similar what had been described in humans.

I speculate that the differences observed in viremia levels among the two NHP experiments after primary infection with DENV-5 serotype could be the result of a strong innate immune response in special TNF-alpha secretion in all animals in the Indian rhesus macaque group as I clearly showed in my data, the same patterned was not observed in the Asian rhesus macaques where I was unable to observed TNF-alpha secretion and all of the animals had viremia levels detected after the first exposure with DENV-5. I also investigated the virus neutralization activity of DENV-5 infected NHP after primary infection, homotypic and heterotypic challenge. I observed a strong

homotypic antibody neutralization response in all animals after primary DENV-5 infection. One of my interesting finding was the fourfold increase in neutralization titers against DENV-5 compared to DENV-4 serotype at day 180 after the primary challenge. This data is relevant as previous NHP studies demonstrated that six months is in agreement what others scientists have showed for cross-neutralization antibodies fading and the detection of only the specific neutralizing antibodies. My data came in agreement that the NHP immune system exposed to newly emerge DENV-5 serotype recognized this virus strain as a different serotype even to the closest related DENV-4 serotype. My NHP data confirm the finding in chapter II, where I described the discovery of new DENV virus as a newly emerged DENV serotype.

***Ae. albopictus* and Ae. aegypti are competent vectors for infection and dissemination of a newly emerged DENV serotype**

This hypothesis was evaluated in chapter IV. I utilized two mosquito species, *Ae. aegypti* and *Ae. albopictus.* The first one was chosen because it is a vector responsible to maintain the DENV in the urban cycle and has being described to be involved in the majority of DENV epidemics. The second one, was used mainly as this vector is believe to maintain DENV in the sylvatic cycle, also *Ae. albopictus* is suggested to be the mosquito responsible to carry DENV from the forest to the urban population, acting as a bridge vector. As I mentioned in my second chapter, this new DENV serotype is genetically close related to DENV-4 serotype. So, I utilized the new emerged DENV-5 and four different strains of DENV-4 serotype. Also, I utilized DENV-5 viremic NHP as a source of blood meal to fed the mosquitoes, considering that artificial blood meal may underestimate vector competence studies. I also utilized Las Cruces NM mosquito strain,

which is a mosquito strain reported to be less susceptible to to DENV-4 infection. I successfully demonstrated that *Ae. albopictus* was able to be infected and disseminate the newly emerged DENV serotype after feeding on viremic NHP. In my vector competence studies using *Ae. aegypti* mosquitoes I showed that DENV-5 presents similar levels of infectivity and dissemination when I compared to sylvatic and human strains of DENV-4 serotype. Differently from *Ae. aegypti* data, two human strains of DENV-4 demonstrated higher percentage of infectivity when compared to DENV-5, although I have not observed difference in dissemination levels among the viruses used in this mosquito species. As I expected, I showed that NIH strain of *Ae. aegypti* had a higher susceptibility to all virus strains tested when compared to *Ae. aegypti* Las Cruses strain. Future studies utilizing caught field mosquitoes especially from geographic region where this virus was isolated but also from different continents regions are necessary to demonstrate that the newly emerged DENV-5 serotype is able to infect and disseminate in *Ae. aegypti* and *Ae. albopictus* vectors contributing for the emergence of this new serotype into the human population.

The newly emerged DENV-5 serotype has particularities in the virus structure compared to the others DENV serotypes what could reflect in host infectivity

Although the reconstruction of different DENV serotypes is similar in many aspects, different serotypes carry distinct characteristic that can reflects the virus function *in vivo* and *in vitro*. I formulated my hypothesis based on the current knowledge about DENV-5 acquired data from chapters II and III where I demonstrated that this new DENV is genetically and antigenic different from the others DENV serotypes by using human and mouse serum and through NHP studies. The diameter of the mature DENV-5

is approximately 500 Å and the virus morphology is a typical morphology of a DENV as previous described. In chapter V, I described the first Cryo-EM reconstruction of a newly emerge DENV serotype at 18 Å resolution. Although, I was able to obtain a very pure and enough virus particles numbers required to performed high-resolution studies using Cryo-EM as a tool, I had logistic issues that impaired the complete success of my aim by the time of my dissertation defense. At 18 Å resolution still is not possible to obtain data that allow any further conclusions about the differences of this new virus and the others serotypes at molecular level. In conclusion, future studies are necessary to obtain a higher resolution of this new serotype, what is extremely relevant to the development of safe vaccines and effective therapeutics as well as infectivity studies.

The production of non-human primate DENV-5 monoclonal antibodies are valuable tool for neutralization studies what could unravel the mechanism of neutralization of DENV as well they can be used as a powerfully instrument for DENV treatment in humans

Non-human primate is relevant specie for DENV studies as they represent the only vertebrate host besides human to be infected in nature. Another important aspect of the valuable role of NHP in dengue studies is their contribution in the elucidation of several aspects of this disease in humans especially because they share in common a close genetic relationship (Wang, Niu et al., 2012). In chapter VI, I showed the production of stable hybridoma using cells from DENV-5 infected NHP. I collected blood; bone marrow and splenocytes from DENV-5 infected NHP and fused with a mouse myeloma cell line. Although the hybridomas originated were stable, they secreted antibodies for only a short period of time. One possible explanation for the occurrence of

this event was probably that the mouse cell line I used in the fusion procedure as have being description in previous studies that mouse myeloma could expel the NHP chromosome soon after fusion procedure. One alternative strategy for future experiments should be the use of a chimera mouse-human heteromyeloma instead a mouse myeloma cell line to fuse with the NHP lymphocytes. Previous studies demonstrated that a mouse-human heteromyeloma produces more stable hybridomas compared to mouse myeloma, based on the rationale that humans and NHP primates are closest then NHP and mouse. Another strategy also could be the immortalization of DENV-5 exposed lymphocytes by using Epstein-bar virus for the production of stable hybridoma cell line.

FUTURE DIRECTIONS AND LONG TERM GOALS

Virologic surveillance of DENV

In the absence of an effective vaccine, the surveillance of DENV is important to the implementation of control measures such as mosquito control in attempt to contain the appearance of large outbreaks. The introduction of a new DENV serotype into the human population and the increase of the *Aedes* mosquito populations can trigger outbreaks of different proportions depending on intrinsic and extrinsic factors of the virus/host/environment to cause the disease.

Here I reported the discovery and characterization of a newly emerged DENV (DKE-121) isolated from a febrile patient in Malaysian state of Sarawak and predicted to be the prototype strain of a new DENV serotype (DENV-5). Since its discovery in 2007, the current status of the emergence of this sylvatic strain into the human population is not known. Hence, one of the future directions that my project leads is the virologic

surveillance in the area where DKE-121 was isolated to determine if DKE-121 has established a human transmission cycle. Additionally, this surveillance is not only important to the detection of DENV-5, but also the emergence of other sylvatic DENV serotypes or re-emergence of the current known viruses. In the absence of constant surveillance, many sylvatic strains and even new serotypes (as example of DENV-5 DKE-121) can be transmitted silently, without our perception. The detection of these viruses would have a great importance in the studies of sylvatic DENV evolution and the dynamic of its appearance into the human cycle. As humans are exploring new areas, many times causing ecological changes that can facilitate their contact with the natural sylvatic reservoirs and vectors deep into the forests, the chances of emergence of new viruses are increased. An active surveillance based on isolation of DENV from the serum of individuals showing symptoms compatible with dengue disease can be a good indicator of the presence of the current serotype(s) circulating in the human population of a specific region. The isolation of DENV from the *Aedes* population also will support the human surveillance and in addition will help in the determination of vector densities, which in turn will inform the necessity of implementation of mosquito control programs.

Vector competence

Studies of vector competence with DENV are important to determine the potential of the virus to establish a sustained transmission cycle into the human population. I have explored the vector competence of domestic (*Aedes aegypti*) and peridomestic (*Aedes albopictus*) mosquitoes using laboratory colonies known to be infected and able to transmit DENV. Both mosquitoes have shown to be suitable competent vectors for DENV-5 DKE-121. The next step will be to investigate if field-caught mosquitoes from

the geographic region where DENV-5 DKE-121 was isolated are also competent vectors for the transmission of the newly emerged serotype. The assessment of infection and dissemination rates of field-caught mosquitoes and comparison with the data from the laboratory strains would help to determine the real potential of DENV-5 DKE-121 been established into the human cycle of that specific Malaysian region. Moreover, it also can provide clues of the dynamic of which mosquito species could be involved as the bridge vector responsible for the transmission of the sylvatic virus into the human population.

Structural studies of DENV-5 DKE-121

Although the reconstruction of different DENV serotypes is similar in many aspects, different serotypes carry distinct characteristic that can reflects the virus function in vivo and in vitro. I my studies I demonstrated the first cryo-electron microscopy (Cryo-EM) reconstruction of DENV-5 DKE-121 at 18 Å resolution, showing that the mature virus has the diameter of approximately 500 Å and the typical morphology of the other DENV serotypes. Further studies to obtain a better resolution would be valuable to show differences at molecular level.

The different stages of DENV life cycle involve major structural changes in the virion. The determination of the protein structure, including tertiary and quaternary structures could reveal important aspects related to host-virus interactions and differences among the other DENV serotypes. Amino acid charges also could explain differences in cell infectivity, as demonstrated the interaction of positively charged residues to heparan sulfate (HS), which serves as a cellular receptor for the virus. Cryo-EM studies are also valuable for the rational design of therapeutics, including vaccines and antiviral drugs. Near atomic resolution could reveal which regions are important for drugs and antibodies

interactions, as well other regions that could be interacting with different cellular receptors of target cells.

Non-human primate monoclonal antibodies against DENV-5 DKE-121

Monoclonal antibodies (MAbs) are valuable tools not only in diagnostics, but also as clinical options to prevent or treat diseases. Antibodies isolated from humans have been used in the treatment of viral diseases, which no specific treatment or vaccine is available. However, due ethical and practical reasons, humans cannot be immunized with all antigens of interest in attempt to isolate therapeutic antibodies. Non-human primates (NHP) are a suitable model that can circumvent the necessity of using human subjects. NHP and human antibodies are shown to have a great similarity. The immunoglobulin (Ig) sequence of NHP has 85-98% homology with the human Ig coded genes. Thus, NHP antibodies have a high degree of tolerance for human treatment.

In my project I have established stable hybridomas using cells from DENV-5 infected NHP. The continuation of this project will involve the selection of hybridomas secreting DENV-specific antibodies, more importantly clones with high affinities and neutralizing properties. Although I have selected a few clones that reacted with DENV-5 antigens, the binding signal was low and the hybridomas lost the ability to secrete the antibody most likely due to the chimerization process. So, a future direction may also involve the use of alternative strategies such as the immortalization of B cells derived from the NHP or the use of a different myeloma cell partner. The isolation of stable hybridomas and production of high affinity antibodies will be valuable not only in future studies to determine the clinical value as a therapeutic compound, but also in studies of the mechanism of virus neutralization. Through the cryo-EM studies, monoclonal

antibodies that bind or neutralize specifically DENV-5 could reveal regions that represent dominant neutralizing epitopes and show differences or similarities among the other DENV serotypes.

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VITA

NAME: Sandra V. Mayer

PRESENT POSITION AND ADDRESS:

Graduate Assistant
Department of Pathology
2.140 Keiller Building
University of Texas Medical Branch
301 University Boulevard
Galveston, TX 77555-0609
E-mail: samayer@utmb.edu or mayer.sv@hotmail.com
Tel: (409) 747-2420 (lab)

BIOGRAPHICAL: Birthplace: Santo Cristo, RS – Brazil
Citizenship: Brazilian

EDUCATION:

1997-2003 Doctor of Veterinary Medicine
Federal University of Santa Maria – UFSM
Santa Maria – RS, Brazil

2003-2005 MSc. Veterinary Medicine
Major: Preventive Veterinary Medicine - Virology
Federal University of Santa Maria – UFSM
Santa Maria – RS, Brazil
Dissertation title: Distribution of Bovine Herpesvirus type 1 (BHV-1) and 5 (BHV-5) DNA in the brain of rabbits during latent infection
Advisor: Eduardo F. Flores

2010-present Experimental Pathology Doctoral Candidate
University of Texas Medical Branch
Galveston, TX
Advisor: Nikos Vasilakis

PROFESSIONAL AND TEACHING EXPERIENCE: (academic; non-academic)

2006-2007 Faculty instructor of Veterinary Microbiology and Public health courses.
University of West Santa Catarina (UNOESC), Xanxere, SC – Brazil.

2012- PATH 6101 - Teaching Skills and Course Development I – UTMB Spring semester.

2012- PATH 6102 - Teaching Skills and Course Development II – UTMB Summer semester.

2012- Teaching Skills Workshop: Why Should Teachers Bother Writing Instructional Objectives? Presented by: Vicki Freeman, May 14, 2012.

2012- Teaching Skills Workshop: Writing Multiple Choice Test Questions. Presented by: Judith F. Aronson, June 19, 2012.

RESEARCH ACTIVITIES:

Area of Research

- Pathogenesis and Epidemiology of Dengue.

FELLOWSHIPS

1999-2002 National Council for Scientific and Technological Development, CNPq - Brazil
Proc. Number: 215/96
Project: PRONEX in Veterinary Virology.
Federal University of Santa Maria – UFSM
Santa Maria – RS, Brazil

2003-2005 National Council for Scientific and Technological Development, CNPq - Brazil
Proc. Number: 130553/2003-4
Project: Latent infection by Bovine Herpesvirus type 1 (BHV-1) and 5 (BHV-5) in experimentally infected rabbits: spontaneous and dexamethasone-induced reactivation and distribution of viral DNA in the central nervous system.
Federal University of Santa Maria – UFSM
Santa Maria – RS, Brazil

2012-2012 James McLaughlin Pre-Doctoral Fellowship
Project: Establishment of animal model to study Dengue Hemorrhagic Fever pathogenesis.
University Of Texas Medical Branch – UTMB
Galveston – TX, USA.

PROFESSIONAL EXPERIENCE:

2005 Visiting Scientist
Department of Population Health
Poultry Diagnostic and Research Center
University of Georgia (UGA)
Athens, GA 30602
- Development of Avian Influenza (AI) ELISA test based on the non-structural (NS) antigen of the virus using a baculovirus expression system.
Advisor: Maricarmen Garcia

2006 Visiting Scientist
Department of Infectious Diseases
Universityof Georgia
Athens, GA 30602
- Expression of recombinant influenza antigens in a baculovirus expression system with the goal of generating virus-like particles (VLPs).
Advisors: S. Mark Tompkins and R. Jeffrey Hogan

2006-2007 Veterinary Diagnostic Lab supervisor and Faculty member
College of Veterinary Medicine
University of West Santa Catarina (UNOESC)
Xanxere, SC – Brazil
- Veterinary Microbiology
- Public health

MEMBERSHIP IN SCIENTIFIC SOCIETIES:

2008 - 2010/2013 AAI - The American Association of Immunologists
2012 - 2016 ASV – American Society for Virology

HONORS/AWARDS:

2010 AAI Travel Grant award for the 14th International Congress of Immunology in Kobe, Japan.
2012 The Edward S. Reynolds Award – Department of Pathology's 18th Annual Research Day. UTMB.
2013 Betty Williams Scholarship Award - Graduate School of Biomedical Sciences, UTMB.
2014: Zhou Sisters Great Expectations Scholarship Award, UTMB.

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MANUSCRIPTS – IN PREPARATION:

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PAPERS AND CONTINUING EDUCATION PROGRAMS PRESENTED:

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Preceptorships:

1. Irma Y. Amaya-Larios, Master student at Centro de Investigaciones sobre Enfermedades Infecciosas, Instituto Nacional de Salud Publica, Cuernavaca, Morelos, Mexico. Plaque Reduction Neutralization Test (PRNT) and DENV propagation and quantification. UTMB, April-July, 2012.

2. Victoria Avanzato, undergraduate student at Pennsylvania State University, State College, PA. Summer Undergraduate Research Program, UTMB. June-August, 2014.