THESIS

OPTIMIZATION AND ANALYSIS OF LIVE ATTENUATED DENVAX-4 CONSTRUCTS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2013

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ABSTRACT

OPTIMIZATION AND ANALYSIS OF LIVE ATTENUATED DENVAX-4 CONSTRUCTS

Dengue virus is a flavivirus that infects millions of people every year, causing high fever and rash and resulting in death in some cases. There are four serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) of dengue virus that are transmitted by the *Aedes aegypti* mosquito, endemic to tropical and subtropical regions of the world. Currently no vaccine for dengue fever is available. The rising number of confirmed cases and the increased habitat of *Aedes aegypti* increase the urgent need for a vaccine. Together with the Centers for Disease Control, Inviragen Inc. has developed a tetravalent live attenuated chimeric vaccine (DENVax) that is currently in phase II clinical trials.

DENVax is based on an attenuated DENV-2 backbone. DENV-2 strain 16681 was passaged 53 times in primary dog kidney (PDK) cells. This strain is the current DENVax-2 strain used in Inviragen's vaccine. There are nine attenuating mutations, three of which are silent. Attenuating phenotypes of DENVax-2 include temperature sensitivity, decreased plaque size, and decreased replication efficiency in mosquito cells. To generate DENVax-1, DENVax-3, and DENVax-4, prM and E genes from wild type DENV-1, DENV-3, and DENV-4 strains were cloned into the infectious cDNA clone of the attenuated DENVax-2 backbone, resulting in chimeras. Tetravalent DENVax has shown significant immunogenic responses in AG129 mice and non-human primates, and is currently in phase II clinical testing.

Data from preclinical tests showed that DENVax-4 is less immunogenic in AG129 mice and non-human primates compared to the other DENVax strains. Two projects in this thesis were completed to reengineer the current DENVax-4 strain to increase immunogenicity. The first project uses blind serial passaging of DENVax-4 first generation and reengineered DENVax-4b second generation as a method to select for strains better fit to grow in vivo. These passaged strains were tested for increased growth kinetics and immunogenicity in both AG129 mice and non-human primates. The second project uses sequencing data from the serial passaging to identify several adaptive mutations in each DENVax-4 construct. These mutations were cloned into DENVax-4 sequence to potentially optimize the strains for Vero cell growth. Three new DENVax-4 constructs were introduced, each containing a different mutation. In addition three new DENVax-4 constructs with wild type reversions of non-critical attenuating mutations were generated. Growth kinetics for all six new DENVax-4 clones were characterized, and testing was done in AG129 mice to determine neutralizing antibody titers.

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CHAPTER 1

DENGUE VIRUS AND DENVAX LITERATURE REVIEW

1.1 DENGUE PREVALENCE

Four dengue virus serotypes exist (DENV-1, DENV-2, DENV-3, and DENV-4) which are capable of causing human disease. Averages of 100 million cases of dengue fever are reported each year, with up to 1 million cases of dengue hemorrhagic fever/ dengue shock syndrome (DHF/DSS) (6). DHF/DSS causes approximately 22,000 deaths per year, the majority of these deaths occurring in children. Dengue virus is transmitted by the mosquito *Aedes aegypti* that lives in the tropical and subtropical regions of the world. Due to the wide-ranging habitat of this mosquito, approximately 2.5 billion people are at risk of developing dengue fever, with more cases being reported annually (6). The number of reported cases since 2000 is twice that from 1990-1999 (Figure 1.1). This is a result of a larger mosquito habitat (attributed to global warming), increased travel, and uncontrolled urbanization in endemic areas, increasing population density. The rise in the number of annual cases of dengue fever greatly exemplifies the need for a vaccine, but currently there are no licensed vaccines available. An ideal dengue vaccine would protect against all four serotypes (to prevent secondary exposure) with only one or two doses, be easy to administer and inexpensive to manufacture, and produce no or minimal clinical symptoms in patients.

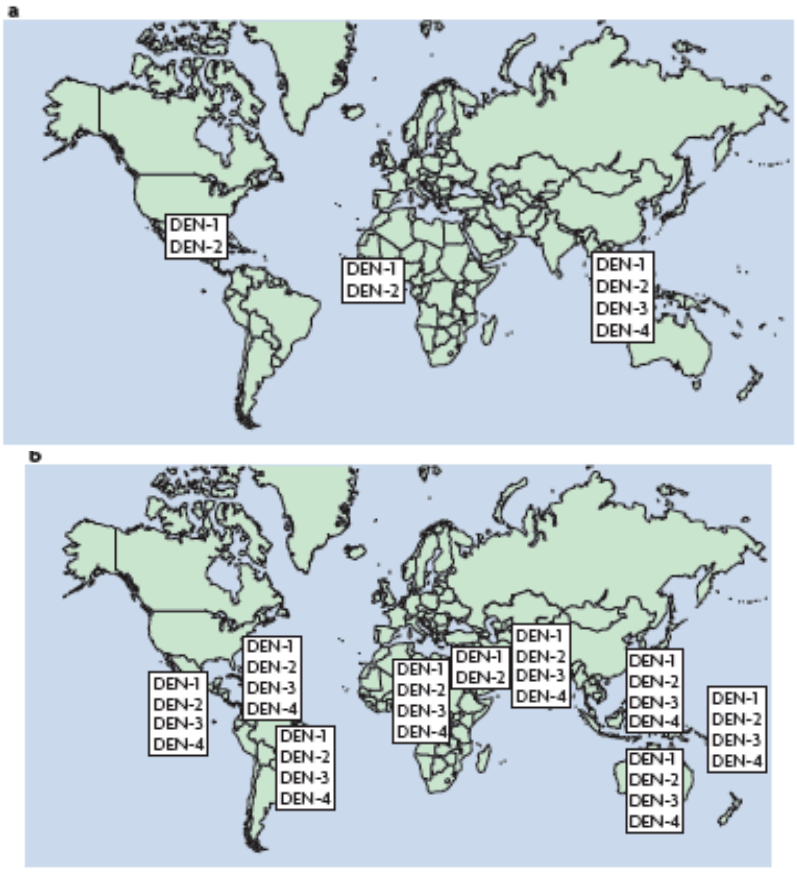


Figure 1.1 **Global distribution of dengue virus serotypes in 1970 (top) and 2004 (bottom)**

Guzman M et al. (2010) Dengue: a continuing global threat. *Nature Reviews*: S7-S16.

1.2 DENGUE VIRUS STRUCTURE

Dengue virus is a flavivirus that is a member of the family *Flaviridae*. The enveloped virus has a positive sense single stranded RNA genome (1) containing a 5' type I cap, but lacks a 3' poly (A) tail. The mRNA has high purine content, and translates into a polyprotein that is cleaved into 3 structural proteins and 7 nonstructural proteins. The DENV structural proteins consist of the capsid, prM, and envelope proteins. The capsid protein is small and is the main component of the nucleocapsid. The protein has a central hydrophobic region with hydrophilic domains at the N and C termini (1). Each capsid monomer contains 4 alpha helices, and the mature capsid protein assembles into a dimer (41). The membrane protein is formed by cleavage of the precursor membrane protein (prM) at position 91 at the C terminus. The prM protein forms a heterodimer with the E protein that is believed to be important for stability during maturation. Upon cleavage of the pr segment the mature virion is released from the infected cell (45). DENV envelope proteins are found on the surface of the viral membrane and have roles in virion assembly, receptor binding, and membrane fusion (Figure 1.2) (4). The E protein has three different domains that play an important role in virus infection, and is the main immunogenic protein of dengue virus (5). Domain I is a beta barrel. Domain II contains the conserved fusion loop that inserts into the endosome membrane during infection. Domain III is responsible for cell receptor binding, and is the primary target for neutralizing antibodies that are very virus specific (40). The Domain III structure also contains the hairpin anchor that attaches the E protein to the viral membrane. This structure consists of two alpha helices separated by a region with a sequence conserved among flaviviruses.

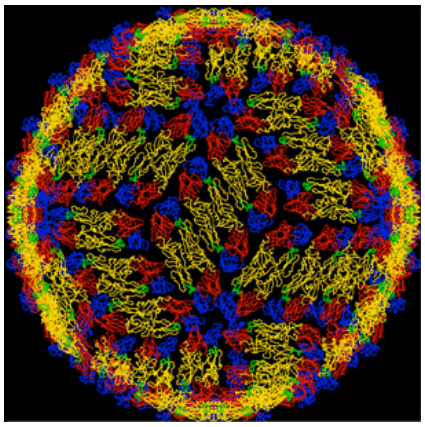


Figure 1.2 **Model of structure of DENV-2.** Structure is based on cryo-EM density and crystal structure of E-protein.

Kuhn RJ et al. (2002) Structure of Dengue Virus: Implications for Flavivirus Organization *Cell* 108(5), 717-725.

The 7 nonstructural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. All the nonstructural proteins play an important role in virus life cycle and infection, mostly because they regulate these processes. NS1 is a beta barrel with amphipathic properties (3). It is synthesized as a monomer during viral replication, but dimerizes in the endoplasmic reticulum and is secreted from infected cells as a hexamer. Although NS1 is essential for virus replication, it is not known what its exact role is during pathogenesis of DENV (42). The NS2 sub-region of the polyprotein contains two small hydrophobic proteins, NS2A and NS2B. NS2A is a protein that spans the membrane of the ER lumen, with the N terminus inside the ER near the C terminus of the NS1 protein. It is believed to contain a peptide sequence that signals for cleavage of NS1 (50). NS2B is a cofactor for the protease domain of NS3 (49). NS3 is highly conserved and has a protease domain and helicase domain (1). The protease domain is at the N terminus and cleaves the NS3/4A, NS4 internal site, and NS4B/5. The helicase domain is at the C terminus and unwinds double stranded RNA and DNA, which is essential for viral replication (43). The NS4 sub-region of the polyprotein contains two small hydrophobic proteins NS4A and NS4B, similar to the NS2 proteins described previously. NS4A colocalizes with RNA during viral replication, and may have an anchoring role (52). The exact role of NS4B is unknown, but studies have shown it associates with NS5 and is involved with the RNA polymerase (51). NS5 has a methyl-tranferase in the N-terminal domain and a RNA-dependent RNA polymerase in the C-terminal domain. It is responsible for replicating and capping viral RNA (43).

1.3 DENGUE VIRUS LIFE CYCLE

Dengue viruses enter host cells through receptor mediated endocytosis, which is initiated by attachment of the E protein to a variety of receptors on the host cell membrane (Figure 1.4). Proposed receptors include lectins and mannose receptors found on macrophages and dendritic cells (53). Once inside an endosome, the low pH triggers irreversible rearrangement of the E protein into homotrimers, after the pre-fusion dimers dissociate to form monomers (3) (Figure 1.5). This exposes fusion loops located in Domain II that initiate fusion of the virus membrane with the endosome membrane through the C-terminal E protein anchor, allowing viral RNA to enter the cytoplasm and begin replication. Once inside the cell the capsid dissociates and the mRNA is released into the cytoplasm. Host-cell machinery is used to translate the polyprotein, which is cleaved by viral proteases upon translation. Viral mRNA for new virus particles is synthesized by the NS5 RNA polymerase. A copy of the mRNA interacts with capsid proteins via the basic domain to form a nucleocapsid precursor (1). Envelope and prM, proteins anchor themselves to the ER membrane through their C termini, with their ectodomains in the ER lumen. New virus particles are budded into the ER lumen once the virus proteins are assembled. These immature virus particles then follow a secretory pathway to the plasma membrane, stabilized by the pr segment of the prM protein bound to the E protein. This inhibits the E proteins from fusing with the membrane of the golgi apparatus. Just before release into the extracellular space the pr segment is cleaved. This is the final maturation step of the virion.

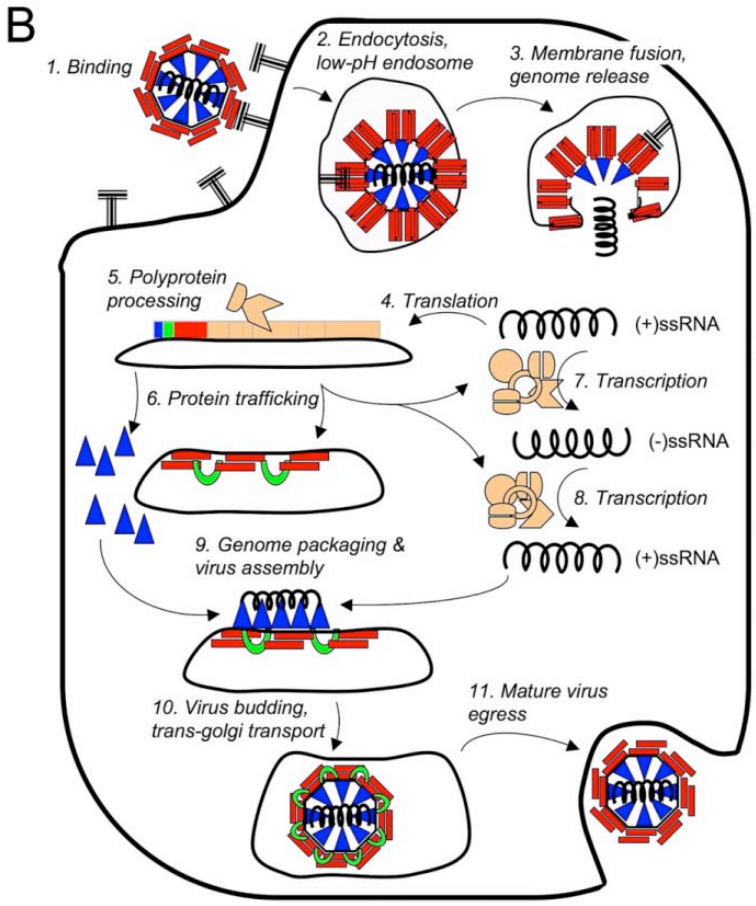


Figure 1.3 **Overview of dengue virus replication and life cycle**. The dengue virion binds to cell surface receptors and enters the cell via endocytosis. Low pH in the endosome causes the E protein to mediate fusion between membranes and release the viral RNA genome into the cytoplasm. After RNA replication and viral protein synthesis immature virions are assembled and budded into the golgi apparatus. Prior to release from the cell the precursor membrane protein segment is cleaved from the membrane protein, producing a mature virion.

Tomlinson SM et al. (2009) New approaches to structure-based discovery of dengue protease inhibitors. *Infectious Disorders Drug Targets* 9, 327-34

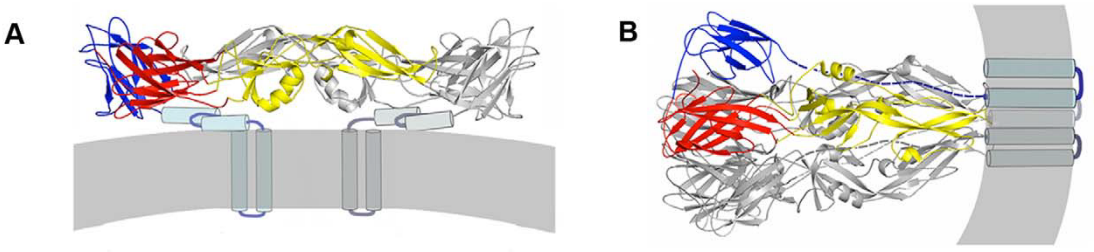


Figure 1.4 **Dimeric structure of the E protein on the virus envelope membrane.** A) Prior to pH change in endosome the E protein lies on the virus envelope membrane in a dimeric structure. B) The reduction of pH in the endosome causes the E protein to "fan out" from the envelope membrane and form a trimeric structure.

Schmidt AG et al. (2010) Peptide Inhibitors of Dengue-Virus Entry Target a Late-Stage Fusion Intermediate. *PLoS Pathogens* 6(4): e1000851

1.4 MOSQUITO REPLICATION CYCLE

Aedes aegypti mosquitoes are endemic to tropical and subtropical regions, commonly found in South and Central America, Africa, India and Southeast Asia (32). Their habitat has recently extended to more temperate zones because of the adaption to reproduce during the warmer seasons. *Aedes aegypti* is believed to have used slave ships in the 15th century to migrate from West Africa to the Americas. The mosquito breeds in stagnant pools, such as in vases or tires filled with rainwater, in urban areas where the large dense populations provide adequate food sources (32). Dengue viruses exist in an urban cycle between humans and *Aedes aegypti* mosquitoes. Dengue viruses replicate in the midgut of the mosquito upon ingestion, which occurs when the mosquito feeds on blood from an infected (and viremic) human (31). Humans that can infect mosquitoes have peak viremia titers in their systems 4-7 days after being bitten by an infected mosquito (37). Ideal replication temperature in mosquitoes is 28°C (31). The virus replicates for 10 days in the mosquito midgut and reaches a peak titer of approximately 9 x 10^3 plaque-forming units per mL (PFU/mL) before disseminating to the salivary glands, where it is further amplified and injected into a host with saliva when the mosquito bites (6). Current studies suggest that *Aedes aegypti* mosquitoes use RNA interference (RNAi) to control virus infection. The RNAi pathway is characterized by recognizing double-stranded RNA (dsRNA) in the cytoplasm of cells infected with RNA viruses. The dsRNA is targeted for degradation by RNAi sensor proteins, which cleave the dsRNA into several short interfering RNAs (siRNAs). One strand of these siRNAs is used by the RNA-induced silencing complex (RISC) to target complementary ssRNA for degradation. This process does not completely inhibit dengue virus replication in *Aedes aegypti*, but studies show that knocking out genes involved in the RNAi

pathway in *Aedes aegypti* decreases the incubation time required for sufficient replication of dengue virus for transmission (31).

1.5 VIRAL REPLICATION AND TREATMENT IN HUMANS

Once a DEN-infected mosquito bites a human, the virus is transmitted with saliva into the skin and bloodstream (6). Virus in the skin infects dendritic cells, which then migrate to the lymph nodes to activate T cells and initiate an immune response. Virus in the blood stream migrates throughout the body and can infect macrophages, monocytes, endothelial cells in the liver, lymphoid tissue in the spleen, and vascular endothelium of the lungs (36). Clinical symptoms of illness usually develop 4-7 days after infection through a mosquito bite, when peak viremia titer is reached. There are three phases to dengue fever; the febrile phase, the critical phase, and the recovery phase (37). During the 5-7 day period of the febrile phase symptoms include high fever, arthralgia and myalgia, nausea and possibly vomiting. The critical phase occurs 3-7 days after development of symptoms. During this phase symptoms of DHF or DSS could occur. The primary symptom of DHF is increased capillary permeability due to low platelet count, causing fluid leakage. Loss of vascular fluids when these symptoms appear results in development of DSS, caused by fluids leaking into the interstitial space. However, most patients do not develop these symptoms and instead proceed directly into the recovery phase. During the recovery phase the fever should subside and platelet count will rise. Some individuals develop a rash during this phase. The best form of treatment for dengue fever is monitoring of symptoms and

administration of fluid IV (37). DENV is not transmissible between humans and cannot be spread by person to person contact. The only preventive method against dengue transmission is mosquito control using mosquito nets, insecticide, and larval habitat reduction. However, with increased mosquito habitats available the mosquito is better able to transmit the virus within human populations and because of the rising mosquito density more humans are at a much higher risk of becoming infected (6).

1.6 VIRUS NEUTRALIZATION AND ANTIBODY DEPENDENT ENHANCEMENT

Infection by one serotype of DENV confers lifelong immunity to that serotype. Infection produces neutralizing antibodies in the host, which are effective at neutralizing the virus if reinfection occurs. Neutralization of DENV is dependent on both antibody affinity and epitope accessibility. Serotype specific neutralizing epitopes are found primarily in Domain III of the E protein. Domains I and II contain weak, non-neutralizing, and/or cross reactive epitopes. Most antibodies produced during infection with a DENV target epitopes in Domain II of the E protein (34). Studies have shown that antibodies which target the fusion loop in Domain II have a higher affinity to immature virions. The large amount of weak antibodies produced also competes with the small amount of strong neutralizing antibodies produced for binding sites. Often the neutralizing antibodies are inhibited by steric interference, contributing to the severity of infection (34). Antibodies can either bind to proteins on the surface of the virion to prevent attachment to cellular receptors or bind to the fusion loop on the envelope protein to prevent

fusion of the virion to the endosome membrane. However, surface antibodies that fail to neutralize virus particles also increase uptake of the virus by monocytes via the Fc gamma receptor (35), possibly increasing the severity of infection.

Neutralizing antibodies are serotype specific, and the host is still susceptible to infection by the other three DENV serotypes after infection with a single serotype. Virus neutralization is one of the most important factors in preventing development of DHF/DSS (37). DHF/DSS more frequently occurs after a secondary infection by a heterologous DENV serotype, and is believed to be the result of antibody-dependent enhancement (ADE). ADE occurs when pre-existing antibodies from a previous dengue infection bind to another dengue serotype but fail to neutralize the virion (7, 8). This complex then binds to monocytic cells bearing Fcγ receptors. Once the complex is taken up by the cells the virus, since it has not been neutralized, can infect the monocytes and cause severe illness. The most frequent cases of DHF/DSS occur in children, since by the time most reach adulthood they have already been exposed to all four serotypes and therefore have complete immunity (6). Recent studies have shown that DHF/ DSS are more likely to occur when primary infection is from DENV-1 or DENV-3, and secondary infection is from DENV-2. Studies have also shown that humans can be infected with more than one dengue serotype and not have symptoms of DHF/DSS (26). However, secondary infection with another dengue serotype is not required to cause DHF/DSS.

1.7 DENGUE VACCINE CANDIDATES

1.7.1 TYPES OF VACCINES

There are several different types of vaccines currently used. Live attenuated vaccines contain a pathogen that has been mutated to decrease its pathogenicity so that it can still replicate in the host, but doesn't cause clinical symptoms (15). Chimeric vaccines use viruses that are built from the genomes of more than one virus. These viruses use an attenuated virus backbone with genes coding for immunogenic proteins inserted. This is a common method to generate vaccines against viruses from the same family. Killed or inactivated vaccines used pathogens that have been killed or inactivated so they don't replicate, but induce an immune response by introducing the host to immunogenic proteins still intact on the pathogen surface. Protein or DNA subunit vaccines consist of only select purified proteins from the pathogen as opposed to the entire molecule. Introducing these proteins into the host induces an immune response to provide future protection against pathogens that express those proteins.

1.7.2 WALTER REED ARMY INSTITUTE OF RESEARCH VACCINE

The Walter Reed Army Institute of Research (WRAIR) developed a live attenuated tetravalent dengue vaccine in partnership with GlaxoSmithKline Biologicals (15). Vaccine viruses were obtained by serial passaging of wild dengue viruses obtained from patients with clinical disease (56). Viruses were passaged in primary dog kidney cells and fetal rhesus lung cells. Two studies,

single-dose and two-dose, were completed with a total of 49 volunteers using monovalent vaccines. Tetravalent vaccine was tested in 10 volunteers using 2 or 3 doses. Yellow fever control vaccine was tested in 4 volunteers. Overall seroconversion rates after a single dose in volunteers immunized with monovalent vaccine were 100% for DENV-1, 92% for DENV-2, 46% for DENV-3, and 58% for DENV-4 (56). Overall seroconversion rates in volunteers immunized with tetravalent vaccine were 100% for DENV-1, 80% for DENV-2, 80% for DENV-3, and 40% for DENV-4. Development of rash was seen in 13 volunteers (22%) and development of fever >100.4 F was seen in 11 volunteers (19%). Most of the clinical symptoms were seen in volunteers immunized with monovalent DENV-1, suggesting under attenuation of the strain. The vaccine has been reformulated to increase attenuation of DENV-1 and decrease attenuation of DENV-4, and is currently in phase II clinical trials.

1.7.3 SANOFI PASTEUR VACCINE

Sanofi Pasteur has developed a tetravalent vaccine that uses the yellow fever vaccine attenuated backbone with prM and E genes for each dengue serotype inserted. Currently the vaccine is in Phase III clinical trials. Results published from the phase II clinical trials show that vaccine efficacy after one dose was 61.2% for DENV-1, 0% for DENV-2, 81.9% for DENV-3, and 90.0% for DENV-4 (48). The sample size was 4002; 2269 children received the dengue vaccine and 1333 received the control. There were 76 confirmed cases of dengue fever after at least one injection. Of the confirmed cases, 52 were DENV-2 infection. The total vaccine efficacy after two injections was 35.3%. These data indicate that further investigation in the DENV-2 vaccine

construct is needed, even though the immunogenicity of the DENV-2 vaccine strain was satisfactory. Sanofi Pasteur is currently investigating whether balanced immunogenicity against all four dengue serotypes is the most effective way to provide immunity (12).

1.7.4 LID/NIAID VACCINE

The Laboratory of Infectious Diseases (LID) at the National Institute of Allergy and Infectious Diseases (NIAID) are using an infectious cDNA clone for an attenuated DENV-4 backbone (15). The clone has 30 base pairs deleted in the 3' untranslated region. This deletion restricts dissemination to the head in mosquitoes, inhibiting virus transmission. Clinical testing in 20 volunteers showed no serious adverse reactions and a 100% seroconversion rate (57). The PrM and E genes were replaced in this backbone with genes from the other 4 serotypes, resulting in three chimeric vaccine strains which are formulated into a tetravalent vaccine. These constructs are currently in preclinical testing.

1.7.5 ARBOVAX INC. VACCINE

Arbovax Inc. has partnered with North Carolina State University to develop a dengue vaccine with attenuation properties in the E protein transmembrane domain (47). The DENV-2 mRNA sequence has large truncations in this domain, which has been shown to decrease replication in vertebrate cells but not in invertebrate cells (i.e. mosquitoes). The large deletions make reversion

to the wild type sequence upon multiple passages very difficult. Three vaccine candidates containing different mutant strains of DENV-2 were tested in African green monkeys and generated sufficient neutralizing antibodies against DENV-2 upon challenge. Constructs for the other three dengue serotypes using this method of attenuation have not been synthesized.

1.8 INVIRAGEN'S DENGUE VACCINE

1.8.1 DEVELOPMENT OF DENVAX-2 BACKBONE

Scientists at Mahidol University in Bangkok, Thailand developed the DENVax-2 backbone that is currently the backbone of Inviragen's dengue vaccine. The candidate DENV-2 vaccine was constructed by isolating DENV-2 from a patient in Thailand with DHF/DSS (strain 16681). The wild type DENV-2 was then passaged in primary dog kidney (PDK) cells 53 times. Cells were grown at 32ºC and passages were done without any deliberate selection. The serial passages caused mutation of the viral genome, which conferred significant attenuation in the RNA genome (22). These attenuation markers were later characterized and mapped by Claire Huang and Richard Kinney at the CDC and included temperature sensitivity, small plaque size and moderate growth in monocytes (20). In 1984 ten adult participants were inoculated with the DENV-2 vaccine candidate. None of the participants showed any symptoms of infection, and adverse reactions were observed at the injection site. All participants developed a neutralizing antibody response 14 days after inoculation that was maintained for up to 18 months after vaccination (54).

1.8.2 IDENTIFICATION AND SIGNIFICANCE OF ATTENUATING MUTATIONS

Scientists at the Centers for Disease Control and Prevention (CDC) in Fort Collins, Co identified 9 attenuation markers/mutations in DENV-2 PDK-53 which differentiate this vaccine strain from the WT 16681 virus (20). Three of these attenuation markers are silent mutations. The remaining six are at 5' NC-57 C-to T, PrM-29 Asp-to-Val, Gly-to-Asp at NS1-53, NS2A-181 Leu-to-Phe, NS3-250 Glu-to-Val, and NS4A-75 Gly-to-Ala. Generation of DENV-2 PDK-53 created two different variants. PDK-53V contains all nine attenuation markers listed above, and PDK-53E contains eight of nine, having wild type NS3-250 Glu. Inviragen's DENV vaccine is based on the PDK-53V backbone.

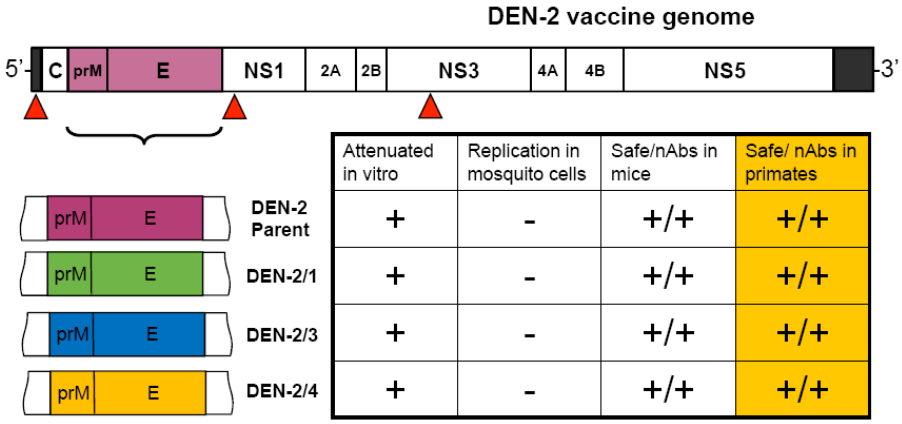
Experiments have shown that the PDK-53 strains have smaller plaque size, greater temperature sensitivity, lower titer, and reduced growth rate (20) as compared to wild type DENV-2. These are all classical signs of attenuation that are important in a vaccine, since the goal of attenuation is to produce a virus that cannot replicate enough to cause clinical symptoms. The PDK-53 strain also shows decreased replication in mosquitoes (20), which will lessen the probability of the vaccine virus from being transmitted. Studies have indicated that 5' NC, NS1, and NS3 mutations contribute to the smaller plaque size and affect virus replication. The NS1 and NS3 mutations also cause temperature sensitivity of the PDK-53 virus. Clinical trials show high immunogenicity in adult Thai participants with no reported symptoms (54).

1.8.3 CONSTRUCTION OF cDNA CLONES

Scientists at the CDC amplified virus stocks of both DENV-2 16681 and DENV-2 PDK-53. The RNA genome was extracted and reverse transcription PCR (RT-PCR) was used to amplify cDNA. The DENV-2 PDK-53V virus is the vaccine strain for dengue 2 (DENVax2). DENVax1, 3, and 4 were created by inserting the genes for the prM/E proteins of each serotype into DENVax2 (19, 21). Infectious cDNA clones were made for each construct using the pBR322 plasmid and PDK-53V. The prM/E genes from the parent DENV strains were cloned into the plasmid (22) (Figure 1.6). DENVax-1 contains prM and E genes from DENV-1 16007 (Thailand 1964). DENVax-3 contains prM and E genes from DENV-3 16562 (Philippines 1964). DENVax-4 contains prM and E genes from DENV-4 1036 (Indonesia 1976). Once amplified the DNA was transcribed into viral RNA and electroporated into cells for virus amplification and sequencing. A few attenuating mutations were noted for each virus. DENVax-1, DENVax-2, DENVax-3, and DENVax-4 were then formulated into a tetravalent vaccine that theoretically protects against all four serotypes (16).

1.9 DENGUE 4 PHYLOGENY

The DENV-4 serotype has undergone much evolution from the original strain introduced into the Western hemisphere in 1981. DENV- 4 was spread first from Indonesia into the Pacific Islands before spreading to North and South America. In 1990 two genotypes were used to classify the



	Attenuated in vitro	Replication in mosquito cells	Safe/nAbs in mice	Safe/ nAbs in primates
DEN-2 Parent	+	-	+/+	+/+
DEN-2/1	+	-	+/+	+/+
DEN-2/3	+	-	+/+	+/+
DEN-2/4	+	-	+/+	+/+

Figure 1.5 **DENVax chimera structure for each serotype.** The prM and E genes from each serotype were cloned into the DENVax-2 PDK-53 backbone to create vaccine strains for each dengue serotype. DENVax-1 (green), DENVax-2 (pink), DENVax-3 (blue), and DENVax-4 (yellow) all showed significant attenuation. Minus signs indicate reduced growth rate in mosquito cells and produced neutralizing antibodies against wild type DENV in both mice and non-human primates.

strains, with strains from east and southeast Asia in one genotype and strains from the Americas in separate genotypes (25). The genotypes were developed based on differences in sequence in the E protein. Studies have shown that while a vaccine strain derived from a genotype two virus provides antibodies against genotypes one and two, a vaccine strain derived from genotype one only provides antibodies against genotype one viruses (25). Recent studies have developed a new phylogenic tree for DENV-4 viruses with three genotypes instead of two as well as a grouping of sylvatic strains believed to infect populations of wild monkeys. The three genotypes are based on entire sequence homology, with more recently evolved strains in genotype three (24) DENVax-4 prM and E genes come from DENV-4 1036, which belongs to genotype two.

1.10 Statement of Projects

While Inviragen's DENV-4 vaccine shows promise to protect humans from DENV-4 infection, improvement is needed to increase immunogenicity, or the level of neutralizing antibody titers. Previous studies have shown that DENVax-4 produces lower neutralizing antibody titers in AG129 mice when compared to the other DENVax strains. This thesis encompasses two projects that focus on further development and testing of DENVax-4. The first project attempts to increase the growth efficiency of the current DENVax-4 strain by blind serial passaging in mammalian cell culture, which is a common method to select for strains more fit to grow *in vivo*. Ten blind serial passages were completed to potentially increase Vero cell adaptation of the DENVax-4 first generation and DENVax-4b second generation vaccines. DENVax-4b contains modifications at the Capsid/prM junction and was previously tested for increased growth

kinetics. Blind serial passaging of DENVax-4 and DENVax-4b produced two new candidate DENVax-4 strains, DENVax-4-P10 and DENVax-4b-P10. An analysis of growth kinetics in Vero cell culture was done on these strains to compare to DENVax-4. Virus was grown in cells for 12 days and samples taken on each day were plaque titrated to measure growth rate and peak titer. Genome sequencing was completed and significant mutations between the P1 and P10 strains were identified in the DENVax-4 and DENVax-4b constructs. Immunogenicity in AG129 mice and non-human primates was also tested and compared to DENVax-4 first generation.

The second thesis project involves incorporating genome changes into the DENVax-4 construct to potentially increase immunogenicity. Previous studies showed that DENVax-4 strains containing wild type DENV-2 backbone instead of the PDK-53 backbone produced higher neutralizing antibody titers. Reversion of the attenuating mutations in the PDK-53 backbone to wild type sequence were introduced into the DENVax-4 genome and tested for increased growth rate in cell culture. In addition, constructs containing mutations noted after the blind serial passaging of DENVax-4 and DENVax-4b were cloned and tested. Growth rate was measured in mammalian cells for 12 days. Samples taken on each day were titrated by immunofocus assay. Growth rate in mosquito cells was also measured to test for attenuation compared to wild type DENV-4. After growth kinetics were completed immunogenicity of selected constructs was tested in AG129 mice. Mice were given a single dose of a designated DENVax-4 construct on day 0, and then challenged on day 56 with DENV-2. Serology and viremia were measured at specific time points, and morbidity and mortality were monitored.

CHAPTER 2

ADAPTATION OF DENVAX-4 BY SERIAL PASSAGES IN VERO CELLS

2.1 BACKGROUND AND RATIONALE

A live attenuated vaccine has a significant advantage over a vaccine containing inactivated pathogens or immunogenic proteins. Live attenuated vaccines tend to elicit stronger and longer lasting immune responses in the host because there is active replication of a pathogen, but the replication efficiency is decreased. This results in no or minimal clinical symptoms produced upon vaccination. The significant advantage of a chimeric vaccine is its ability to elicit a strong immunogenic response in the host against multiple strains or serotypes of a virus while using a single backbone as the vaccine platform, eliminating the need to attenuate multiple virus strains Dengue virus has 4 different serotypes. Development of a vaccine against all 4 serotypes is made easier by developing a single attenuated backbone and inserting genes to ensure that immunogenic proteins from all 4 dengue serotypes are expressed. During the preclinical research and development of DENVax-4 three different chimeras were produced. All three chimeras contain the prM and E genes from DENV-4 1036, but contain different backbones. D2/4-P contains the DENV-2 strain 16681 backbone, D2/4-E contains the PDK-53-E backbone (which has all but one of the attenuating mutations found in the DENVax-2 backbone), and D2/4-V contains the PDK-53-V backbone, which contains all the attenuating mutations found in DENVax-2 and is the construct used to create the current DENVax-4 strain. These three chimeras were tested for immunogenicity in AG129 mice and compared with the wild-type

DENV-4 1036. The D2/4-P chimera showed similar immunogenicity to DENV-4 1036, but antibody titers in mice injected with D2/4-E or D2/4-V were significantly reduced (21). These results indicate that modification of the DENVax-4 clone may be needed to improve immunogenicity in AG129 mice. The hypothesis tested by a previous graduate student at Inviragen was to modify the current DENVax-4 strain to contain a capsid/prM junction to be more genetically similar to DENV-4 instead of DENV-2 to potentially improve replication efficiency of the virus. The current strain of DENVax-4 has a capsid/prM sequence that is identical to DENV-2 instead of DENV-4, creating a RNA secondary structure different than that of DENV-4 (55). The difference in RNA secondary structure affects RNA stability and function. Inserting these modifications in the capsid/prM sequence to make the sequence similar to DENV-4 instead of DENV-2 may improve the RNA stability and translation by inducing a RNA secondary structure more like that of DENV-4, and possibly increase DENVax-4 replication i*n vitro* and *in vivo*.

A previous graduate student at Inviragen produced three new DENVax-4 constructs (DENVax-4b, DENVax-4c, and DENVax-4d) by designing synthetic fragments containing the modifications for each construct and cloning them into the DENVax-4 plasmid (55). DENVax-4b contains 7 total amino acid changes, DENVax-4c contains 9 total amino acid changes, and DENVax-4d contains an amino acid deletion at capsid position 93 (Figure 2.1). RNA was

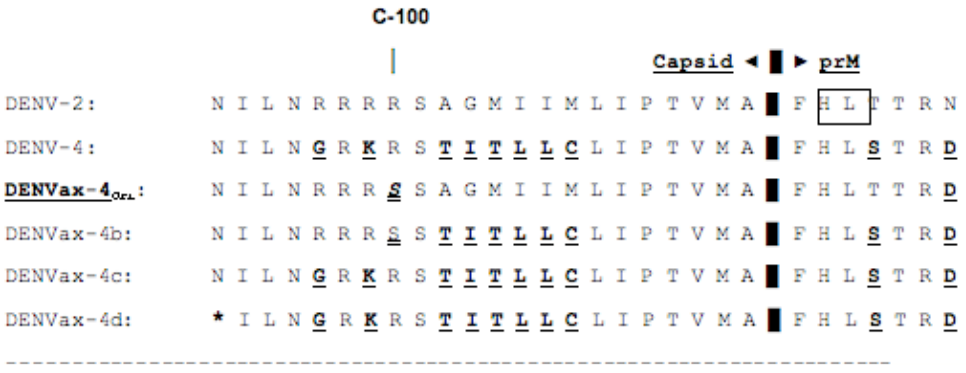


Figure 2.1 **Modifications done to the Capsid/prM junction in each of the DENVax-4b, 4c and 4d constructs**. Changes were made in the amino acid sequence to synonymize with the DENV-4 sequence instead of DENV-2 sequence. In the capsid gene, DENVax-4b has six amino acid changes, DENVax-4c has eight, and DENVax-4d has eight changes and one deletion. All three constructs have two amino acid changes in the prM gene.

transcribed and electroporated into Vero cells for virus rescue. DENVax-4d did not grow to sufficient titers after electroporation, and was not used in future experiments. DENVax-4b and DENVax-4c were tested for growth efficiency in Vero and C6/36 cells in a growth kinetics experiment, with DENVax-4-P2, DENVax-4-P8 and DENVax-2-P2 used as controls. DENVax-4-P2 and DENVax-2-P2 are virus samples that have not been genotypically selected by viral plaque purification, a process in which individual viral plaques are picked from a DENVax-infected Vero cell monolayer and then over-laid with agarose gel containing neutral red to visualize single plaques. DENVax-4-P8 had been selected by plaque purification to obtain a virus stock with a clonal DENVax-4 genotype. This procedure is done to generate a master seed virus with no reversion of attenuating mutations. DENVax-4c did not reach an adequate peak titer after growth in Vero cells, and had a slower initial growth rate than either DENVax-4 or DENVax-4b (Figure 2.2). There was no significant difference between the peak titers of DENVax-4 and DENVax-4b in the Vero growth curve, and both had similar initial growth rates (Figure 2.2). In the growth analysis in C6/36 mosquito cells, DENVax-4b and DENVax-4c both reached peak titers that were significantly less than wild type DENV-4, confirming their attenuation (Figure 2.3).

Based on the data from this study, further reengineering of the DENVax-4 construct is needed to potentially increase immunogenicity. Passaging in cell culture is a classic method of selecting for virus strains with adaptive mutations for increased replication rates in vivo, and was one of the principal methods in developing the DENVax-2 attenuated backbone. The Vero growth curve also showed that DENVax-4-P8 had a faster initial growth rate than DENVax-4-P2. In addition to

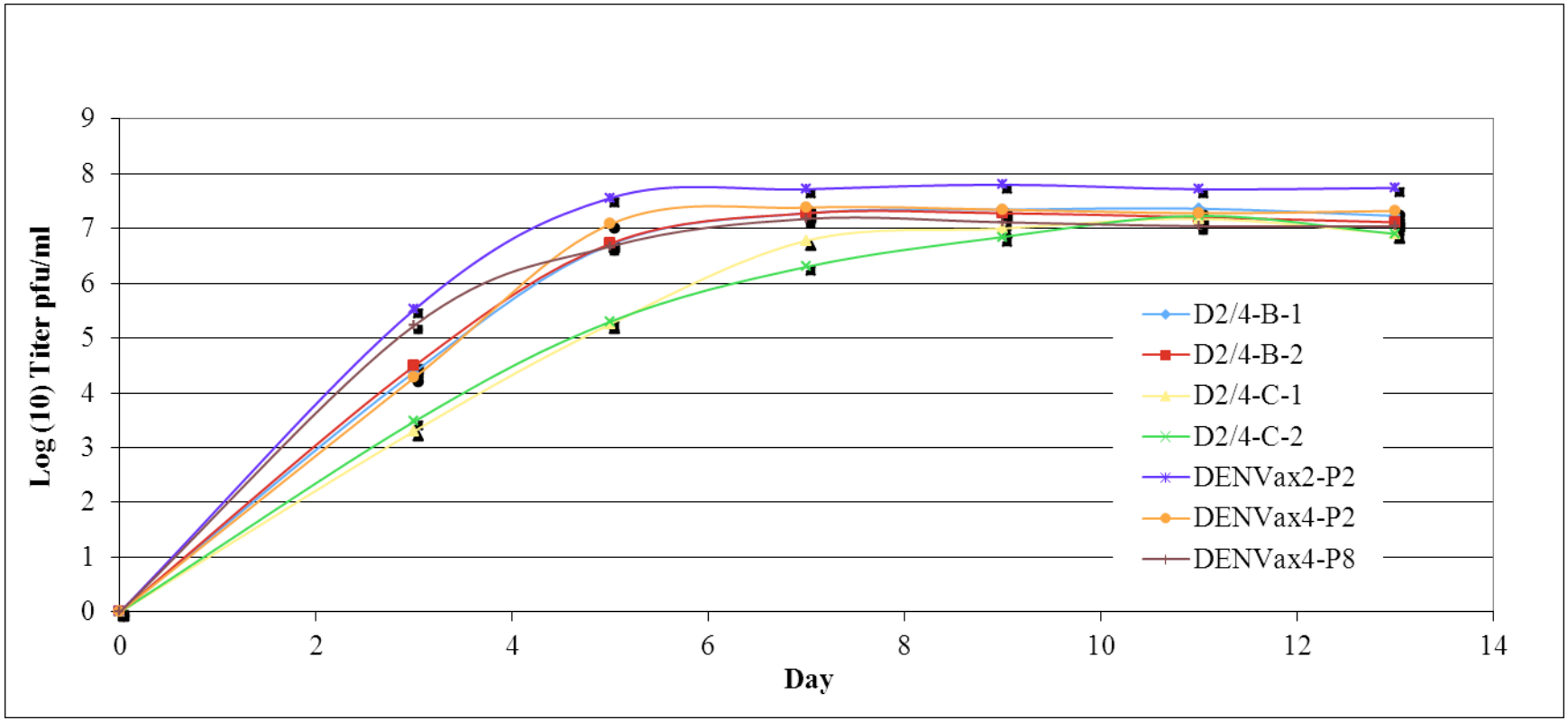


Figure 2.2 **Growth curve of the modified DENVax-4 constructs in Vero cells**. New DENVax-4 second generation constructs (D2/4-B and D2/4-C) were compared in duplicate with first generation DENVax-4-P2, DENVax-2-P2, and plaque purified DENVax-4-P8. The day each sample was taken is on the x-axis and the titer of the sample is on the y-axis.

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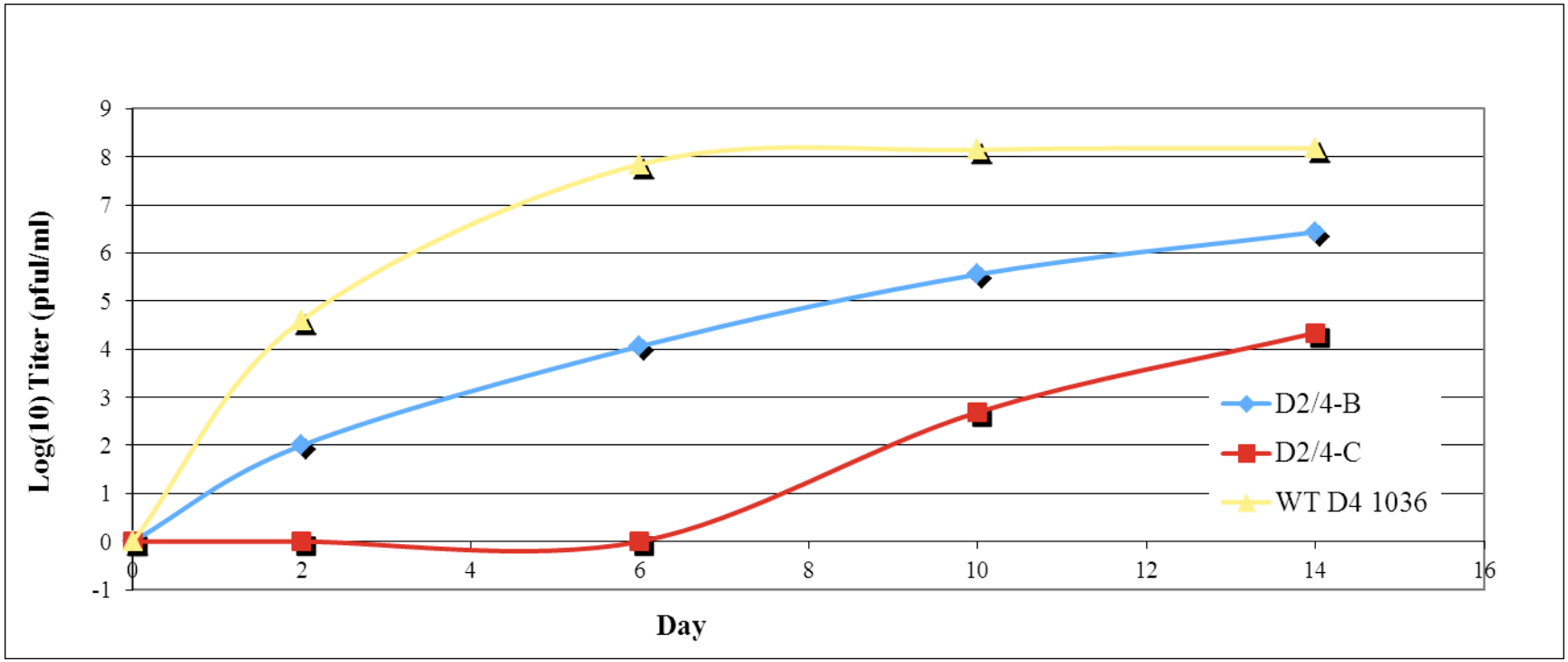

Figure 2.3 **Growth curve results of DENVax-4 modified constructs in C6/36 mosquito cells.** New DENVax-4 second generation constructs (D2/4-B and D2/4-C) were tested for decreased growth rate in mosquito cells compared to wild type DENV-4 1036. The day each sample was taken is given on the x-axis and the titer of each sample is on the y-axis.

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being plaque-purified, DENVax-4-P8 had been passaged six additional times in Vero cells compared to DENVax-4-P2, suggesting that adaptation of the vaccine strain in Vero cells could increase growth efficiency. The hypothesis is that if DENVax-4 and DENVax-4b are both adapted to Vero cells by subsequent passaging *in vitro*, mutations will be introduced into the DENV genome that select for higher replication efficiency. In this study, DENVax-4 and DENVax-4b were blindly passaged 10 consecutive times in Vero cells with the goal of adapting the viruses to grow more efficiently in Vero cells. This adaptation allowed us to identify mutations which directed the design of two new vaccine candidate strains, DENVax-4-passage 1(P1) and DENVax-4b-P10. Growth kinetics of the resulting strains were analyzed in a Vero growth curve experiment. The passage 1 and passage 10 strains were sequenced to identify mutations that occurred as a result of serial passaging.

2.2 MATERIALS AND METHODS

2.2.1 CELL CULTURE

Vero cells are mammalian cells derived from African green monkey kidney. The Vero cell line used in the in vitro experiments was obtained from Inviragen's vaccine certified cell bank, produced at Waisman Manufacturing, Madison, WI. Vero cells were grown at 37ºC with 5% CO_2 in Dulbecco's modification of Eagle's Medium (DMEM, Mediatech Inc., Manassas VA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan UT), 2% L-glutamine (200 mM) (Hyclone), and 1% penicillin-streptomycin (Pen-Strep, Hyclone). To passage the cells Tryple Express solution (1X laboratory derived trypsin) (Life Technologies, Grand Island NY)

was used to remove the cells from the flask surface. Cells were diluted 1:24 for passage once a week, and passaged 20 times before new cells were thawed.

2.2.2 VIRAL INFECTION OF VERO CELL MONOLAYERS

Vero cells ($5x10^5$ cells/mL) were seeded on T-75 cm^2 flasks approximately 48 hours before infection. DMEM supplemented with 10% FBS, 2% L-glutamine, and 1% Pen-Strep was used as cell growth medium. Upon confluence 1 flask was treated with 4 mL of Tryple Express solution (laboratory synthesized trypsin) to detach cells from the surface. Cells from the flask were counted to establish a multiplicity of infection (MOI) at 0.001. Two of the remaining flasks were infected with 1 mL of either DENVax-4-P2 (first generation DENVax-4) or DENVax-4b-P3 diluted in BA-1 diluent (bovine serum albumin, 1X M199, 0.05M tris-HCl, 1X L-glutamine, 7.5% sodium bicarbonate, 1X pen-strep, 1X fungizone). Viruses were adsorbed onto Vero cells for 90 minutes with rocking every 10 minutes to prevent drying of cell monolayers. After adsorption 20 mL DMEM supplemented with 5% FBS were added to each flask without removal of inoculum. Flasks were incubated for 7 days at 37°C.

2.2.3 VIRAL HARVESTS AND SUBSEQUENT INFECTIONS: BLIND PASSAGE

On Day 7 of virus growth, the CPE was observed on each flask and virus-containing supernatant was harvested and stabilized in 20% FBS for storage at -80ºC. Previously seeded confluent T-75

cm^2 Vero flasks were infected with 1 mL of the viral supernatant from the preceding flask. Virus was adsorbed for 90 minutes with rocking every 10 minutes. After viral adsorption, 20 mL DMEM 5% FBS was added to each flask. New non-infected control flasks were plated every 7 days. This process was repeated every 7 days for 10 subsequent weeks, yielding 10 passages per virus denoted either DENVax-4-P1 through P10 or DENVax-4b-P1 through P10.

2.2.4 PLAQUE TITRATION OF VIRUSES

Harvests of DENVax-4-P2 and DENVax-4b-P3 from weeks 1, 5 and 10 were plaque titrated to measure infectious virus titer. Virus samples were serially diluted $1x10^{-1}$ to $1x10^{-6}$ in BA-l diluent. Samples were plaque titrated in triplicate, and 100 µL of each dilution was adsorbed to a pre-seeded 6-well plate of Vero cells for 90 minutes with rocking every 8 minutes. After adsorption wells were overlaid with 4 mL of a 1:1 mixture of balanced salt solution (BSS) (NaCl, KCl, NaH_2PO_4-H_2O, glucose, $CaCl_2$-$2H_2O$, $MgSO_4$-$7H_2O$) and1.6% agarose solution and incubated for 4 days at 37ºC. Inoculum was not removed prior to overlay. On day 4 wells were over-laid with 2 mL BSS/1.6% agarose solution supplemented with neutral red (MP Biomedicals, Santa Ana, CA) and incubated overnight at 37ºC. Neutral red solution is adsorbed only by living cells, so virus plaques with dead cells will be visible against the red cell monolayer. Plaques were counted on days 5, 6 and 7.

2.2.5 GROWTH CURVE ANALYSIS

The growth kinetics of the adapted strains were analyzed by performing a growth curve on Vero cells. Vero flasks were seeded as previously described. On day 0 a confluent flask of Vero cells was counted to calculate the virus PFU needed to infect the flasks at an MOI of 0.001. Flasks were infected with 1 mL of DENVax-4, DENVax-4b-P1, DENVax-4b-P10, or DENVax-2. Viruses were adsorbed to the monolayers for 90 minutes with rocking every 8 minutes. After adsorption, 10 mL DMEM without FBS supplemented with 1% F-127 (Inviragen patented product that enhances viral growth) (DMEM-F127) was added to each flask and the samples were incubated at 37ºC. Samples were collected from the supernatant from each flask on day 2 and days 4-12. Viruses were harvested by collecting the entire amount of the supernatant in the flask, and the growth medium was replaced with fresh DMEM-F127 medium on day 4 and days 6-12. Flasks were washed 3 times with phosphate buffered saline (PBS) during medium changes. Samples were stabilized in 1x FTA (15% trehalose, 1% F-127, 0.1% human serum albumin, PBS) and plaque titrated as previously described to determine titer.

2.2.6 SEQUENCING

To identify mutations accumulated during serial passaging, viral RNA from DENVax-4-P1 and P10 and DENVax-4b-P1 and P10 was sequenced at the CDC. Viral RNA was isolated from virus stocks using a QIAmp viral RNA kit. Reverse transcription PCR (RT-PCR) was used to transcribe the RNA into DNA, using primers previously designed by Claire Huang and Richard

Kinney at the CDC. The primers are based on the genome sequences of DENV-2 16681 and DENV-4 1036. Approximately 7-9 DNA fragments per construct were amplified through RT-PCR. The DNA fragments were then sequenced by Beckman Coulter Genomics (Danver, MA) using an automated sequencing reaction, and aligned for comparison.

2.2.7 IMMUNIZATION IN AG129 MICE

To test for immunogenicity DENVax-4b-P1 and DENVax-4b-P10 were inoculated in AG129 mice. There were 4 groups of mice (DENVax-4, DENVax-4b-P1, DENVax-4b-P10, and FTA) with 10 animals per group. On day 0 mice were subcutaneously injected with either 10^5 PFU/mL virus dose or FTA. Mice were boosted on day 42 and bled on days 21, 42, and 56 for serology. Morbidity and mortality were monitored for the duration of the study. All animal testing was done at Inviragen in Madison, WI. Mouse sera samples were sent to Inviragen in Fort Collins for analysis. Neutralizing antibody titers were determined by plaque reduction neutralization test.

2.2.8 PLAQUE REDUCTION NEUTRALIZATION TEST

To test for neutralizing antibodies against DENV-4 in sera samples, a plaque reduction neutralization assay was used. Vero 6-well plates were seeded 2 days before inoculation to ensure monolayer confluence. Serum samples were diluted serially two-fold in BA-1 diluent in a 96-well plate and incubated with DENV-4 for approximately 20 hours at 4ºC. After incubation

Vero wells were inoculated with prepared virus/serum dilutions. Samples were adsorbed for 90 minutes with rocking every 8 minutes to prevent drying of the monolayers. After adsorption wells were over-laid with 1:1 solution of BSS and 1.6% agarose, and incubated at 37ºC for 4 days. On day 4 cells were over-laid with a 1:1 ratio of BSS/ 1.6% agarose solution supplemented with neutral red. Plaques visible on wells were counted on days 5, 6, and 7.

2.2.9 NON-HUMAN PRIMATE STUDY

Testing in non-human primates (NHP) was performed by our colleagues at the Inviragen facilities in Madison, WI. Cynomolgus macaques were placed in 5 different study groups and vaccinated with varying doses of tetravalent DENVax. There were 5 different vaccine formulations and 1 formulation per group was tested. Formulation 1 contained a high dose (10^5) of DENVax with DENVax-4 first generation. Primates in Group 1 were inoculated with 2 doses on day 0 and given no boost. Formulation 2 contained a high dose of DENVax with DENVax-4b-P10. Primates in Group 2 were inoculated with 2 doses on day 0 and given no boost. Formulation 3 contained increased DENVax-4 in the high dose DENVax (10^6). Primates in Group 3 were inoculated with 2 doses on day 0 and given no boost. Formulation 4 contained increased DENVax-4 in the high dose DENVax. Primates in Group 4 were inoculated with 1 dose on day 0 and boosted with 1 dose on day 60. Formulation 5 contained increased DENVax-4 and decreased DENVax-2 in the high dose DENVax. Primates in Group 5 were inoculated with one dose on day 0 and boosted with 1 dose on day 60. The vaccine was administered sub-cutaneous by using a needle and syringe. Serum samples were taken on days 0, 28, 58, 73, 90,

128. Neutralizing antibody responses in sera were measured by plaque reduction neutralization test.

2.3 RESULTS

2.3.1 CYTOPATHIC EFFECTS DURING SERIAL PASSAGES

Cytopathic effects (CPE) describe the change in appearance of cells after infection with a virus. The infected cell is stressed producing large amounts of virus and has abnormal organelle function and decreased replication. As a result the cell will detach from the flask and most eventually die by apoptosis. Viruses cause different CPE in various cell lines. Attenuated dengue viruses do not generate much CPE in Vero cells when grown *in vitro*, but the presence of CPE can indicate successful virus infection and high titer growth. Classical CPE appearance of dengue infected Vero cells are cell rounding and floating. Infected cells will "shrivel" into a smaller round cell. These cells are termed "rounders". Rounders will eventually detach from the flask and float in the medium. These cells are termed "floaters". CPE is analyzed by estimating the percentage of the cells that have become rounders and floaters. CPE was observed weekly on each flask before harvest of media and graded using the qualitative Vero cell culture CPE scale (Table 2.1). While both flasks showed high numbers of floaters, DENVax-4-P2 showed more floaters than DENVax-4b-P3 (Table 2.2). Both flasks showed similar high numbers of rounders. There was no cell detachment on any of the flasks. Control flasks showed high numbers of rounders due to overgrowth.

Table 2.1 **Cell CPE scale used to grade expression of CPE on Vero cell monolayers**. "Rounders" (R) refers to cells that have balled up but not completely detached from the flask. "Floaters" (F) refers to the rounded cells that have detached from the flask.

Scale	Cytopathic Effects (CPE)
-	No CPE, no or very few rounders/floaters
F+	No CPE, some floaters/rounders
F1	Floaters= low density
F2	Floaters= intermediate density
F3	Floaters= high density
F4	Floaters= very high density
R1	Rounders= low density
R2	Rounders= intermediate density
R3	Rounders= high density
1	Cells detach, clear 1-5% of plastic
2	Cells detach, clear 6-15% of plastic
3	Cells detach, clear 16-30% of plastic
4	Cells detach, clear 31-50% of plastic
5	Cells detach, clear >50% of plastic
6	Cells detach, clear 100% of plastic

Table 2.2 **CPE of DENVax-4 and DENVax-4b in Vero cells.** CPE was measured weekly during blind serial passaging. Cells were graded according to the Vero CPE scale (Table 2.1) by estimating the number of rounders and floaters in the cell medium and the percent of cells detached from the flask.

Passage	DENVax-4 CPE+	DENVax-4b CPE+
1	F1, R1	F1, R1
2	F2, R3	F2, R3
3	F3, R3	F3, R3
4	F3, R3	F4, R3
5	F2, R3	F3, R3
6	F2, R3	F2, R3
7	F3, R3	F2, R3
8	F3, R3	F2, R3
9	F3, R3	F4, R3
10	F3, R3	F3, R3

2.3.2 GROWTH KINETICS

DENVax-2 was included as a control and displayed the highest initial titer at day 2, followed by DENVax-4b-P10, DENVax-4, and DENVax-4b-P1 (Figure 2.4). At the end of the growth period (day 12) DENVax-2 had the highest peak titer, followed by DENVax-4b-P10, DENVax-4b-P1, and DENVax-4. However, the calculated differences in titer in all the DENVax-4 strains are too small to be significant (Table 2.3). Like many biological assays, plaque assays have an inherently large standard deviation, and can range to +/-0.5$\log_{10}$PFU. Although the graphs indicate a slight growth advantage of the DENVax-4b-P10 strain, these differences may not be significant.

2.3.3 AMINO ACID CHANGES IN SEQUENCES

DENVax-4-P10 genome sequencing showed mutations that corresponded to amino acids Envelope E417K and NS4A M17L. DENVax-4b-P10 genome sequencing showed a mutation that corresponded to amino acid Capsid C107Y. The Envelope E417K mutation changes the amino acid residue so that a side chain with an amine group (NH_2) is substituted for a side chain with a carboxyl group. The R group remains hydrophilic, but the residue is more basic and has a positive charge at neutral pH. The NS4A M17L mutation results in removal of a sulfate from the R group, but maintains non-polarity resulting in a hydrophobic amino acid. The Capsid C107Y mutation results in drastic change in the R group. Cysteine has an SH group that is capable of

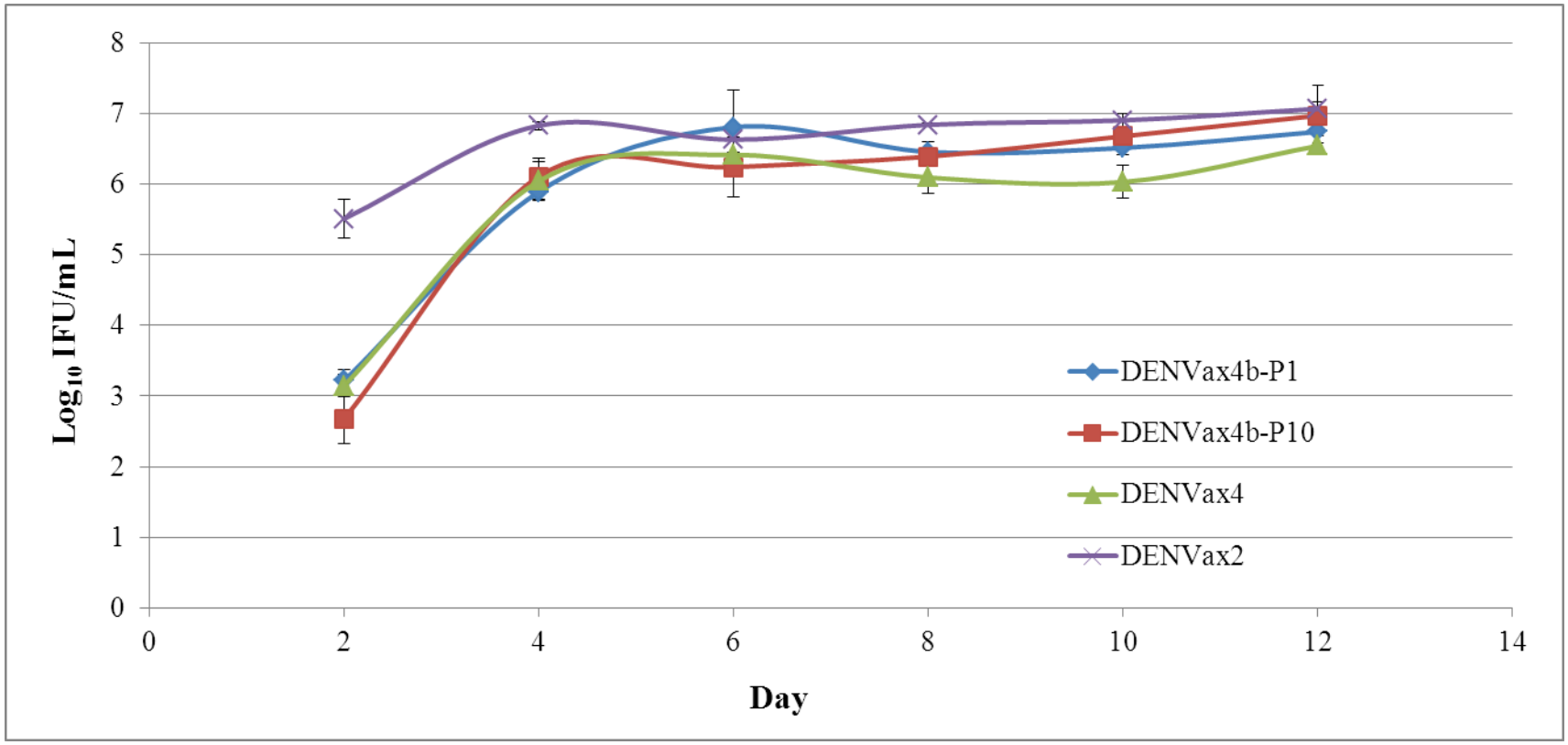

Figure 2.4 **Growth curves of DENVax-4 constructs during growth kinetics experiment.** DENVax-4 and DENVax-4b passage 10 viruses were tested for an increase in growth rate compared to1st generation DENVax-4. DENVax-2 was included as a control. The day each sample was taken is plotted on the x-axis and the titer is plotted on the y-axis.

Table 2.3 **Average titers for DENVax-4-P10 and DENVax-4b-P10 growth curves.** Titers are given in PFU/mL. Standard deviations were calculated using the titers of four replicates of each sample.

	DENVax4b-P1	**DENVax4b-P10**	**DENVax4**	**DENVax2**
Day	**Titer +/- Std Dev**	**Titer +/- Std Dev**	**Titer +/- Std Dev**	**Titer +/- Std Dev**
2	3.22+/-0.16	2.68+/-0.34	3.15+/-0.16	5.51+/-0.27
4	5.89+/-0.11	6.11+/-0.25	6.04+/-0.27	6.83+/-0.06
6	6.80+/-0.53	6.24+/-0.42	6.41+/-0.23	6.63+/-0.19
8	6.45+/-0.14	6.39+/-0.03	6.09+/-0.22	6.84+/-0.03
10	6.51+/-0.10	6.68+/-0.09	6.03+/-0.24	6.90+/-0.10
12	6.74+/-0.06	6.97+/-0.20	6.54+/-0.04	7.07+/-0.33

forming disulfide bonds, while tyrosine has a carbon benzene ring with a hydroxyl group. This causes the amino acid residue to become hydrophilic instead of hydrophobic, affecting its interaction with the other amino acid R groups.

2.3.4 IMMUNOGENICITY IN AG129 MICE

Samples taken on days 21 and 41 were analyzed by PRNT to determine neutralizing antibody response after a single immunization on day 0. Sera samples from day 56 were analyzed to determine neutralizing antibody titers after boost on day 41. DENVax-4 first generation had the highest neutralizing antibody response on day 56 after immunization (Table 2.4). DENVax-4b-P10 had the highest neutralizing antibody response on Day 21, but the response significantly decreased on Days 41 and 56. DENVax-4b-P1 had no significant neutralizing antibody titers after primary or secondary immunizations with titers not significantly different from the control FTA background titer levels. Because of possible introduction of inconsistencies during sample testing, the study was dismissed and further investigations into DENVax-4 modification were based on the sequencing results of the blind Vero passages.

Table 2.4 **Geometric mean titer (GMT) values of AG129 sera samples.** Day 21 serology was completed to measure neutralizing antibody response after immunization on day 0. Day 56 serology was completed to measure neutralizing antibody titers after immunization plus vaccine boost on day 42. GMT values were calculated by averaging the 50% neutralization dilution of each mouse in the group.

	Day 21	**Day 56**
	GMT +/- Standard Deviation	**GMT +/- Standard Deviation**
DENVax-4	44.4 +/- 11.4	285.1 +/- 187.1
DENVax-4b-P1	44.4 +/- 39.5	35.6 +/- 82.1
DENVax-4b-P10	160.0 +/- 105.5	78.2 +/- 66.0
FTA	33.6 +/- 33.6	14.5 +/- 44.6

2.3.5 NEUTRALIZING ANTIBODIES IN NHP VACCINATED WITH DENVAX

The evaluation of the second generation DENVax-4 viruses in NHP took place with a larger study testing immunization regimens. I will focus on the groups immunized with the adapted vaccine strains described above. The immunogenicity of DENVax-4b compared to that of DENVax-4 was tested. Groups 1 and 2 were vaccinated on day 0 with 2 doses and given no booster vaccination. Equivalent titers of either DENVax-4 or DENVax-4b were used. No significant differences in geometric mean titers (GMT) of neutralizing antibodies were found between any of the serotypes including DENV-4 (Figure 2.5). This suggests that using DENVax-4b in tetravalent DENVax does not affect or increase the neutralizing antibody response against DENV-4.

Neutralizing antibody responses in Groups 1 and 3 were compared to determine whether increasing the dose of DENVax-4 in tetravalent DENVax increased immunogenicity against DENV-4. Results showed that primates immunized with a higher dose of DENVax-4 showed an increase in GMT of primary neutralizing antibodies detected in the first 60 days compared to those immunized with traditional tetravalent DENVax (Figure 2.6). There was no significant difference in GMT between the other DENV serotypes. This suggests that a higher dose of DENVax-4 improves the neutralizing antibody response against DENV-4.

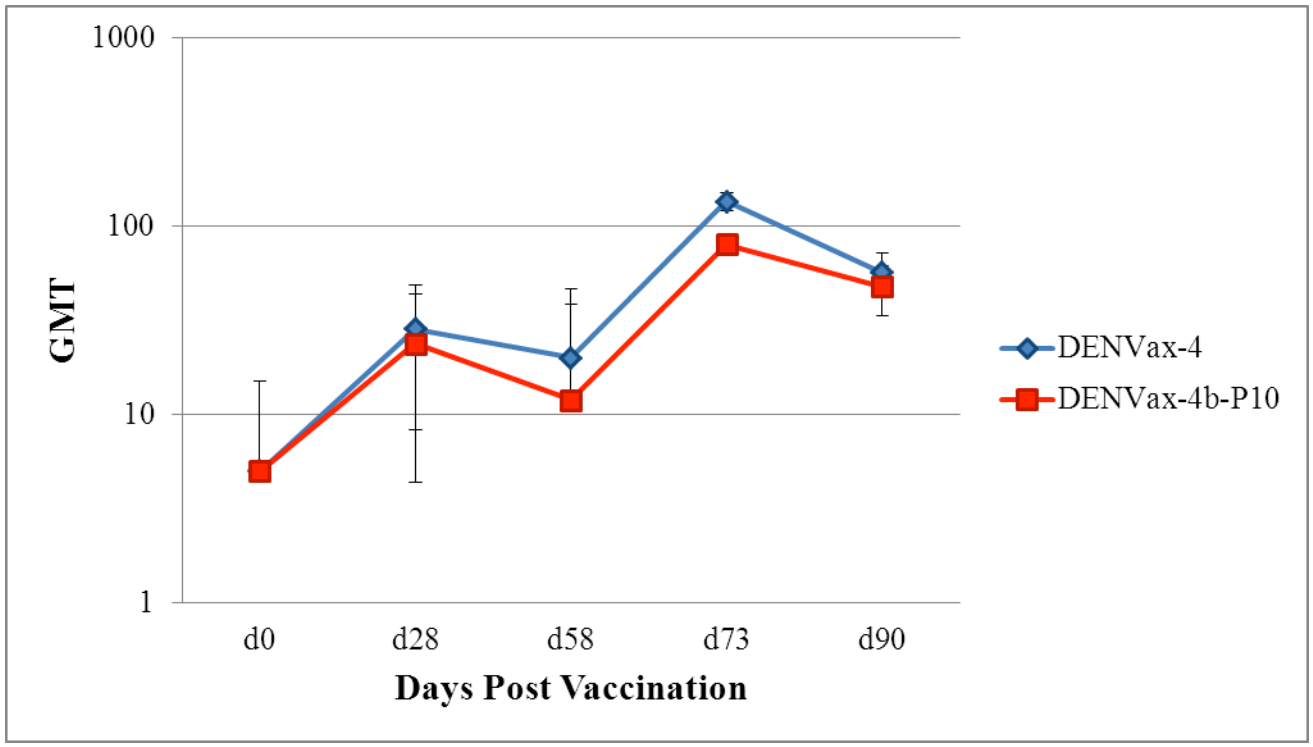

Figure 2.5 **Immunogenicity of DENVax-4-P10 and DENVax-4b-P10 in non-human primates.** Vaccine formulations were tetravalent containing either first or second generation DENVax-4. GMT values are measurements of neutralizing antibody titers from Plaque Reduction Neutralization Technique with DENV-4. DENVax-4-P10 values (blue) and DENVax-4b-P10 values (red) were measured at four time points post vaccination.

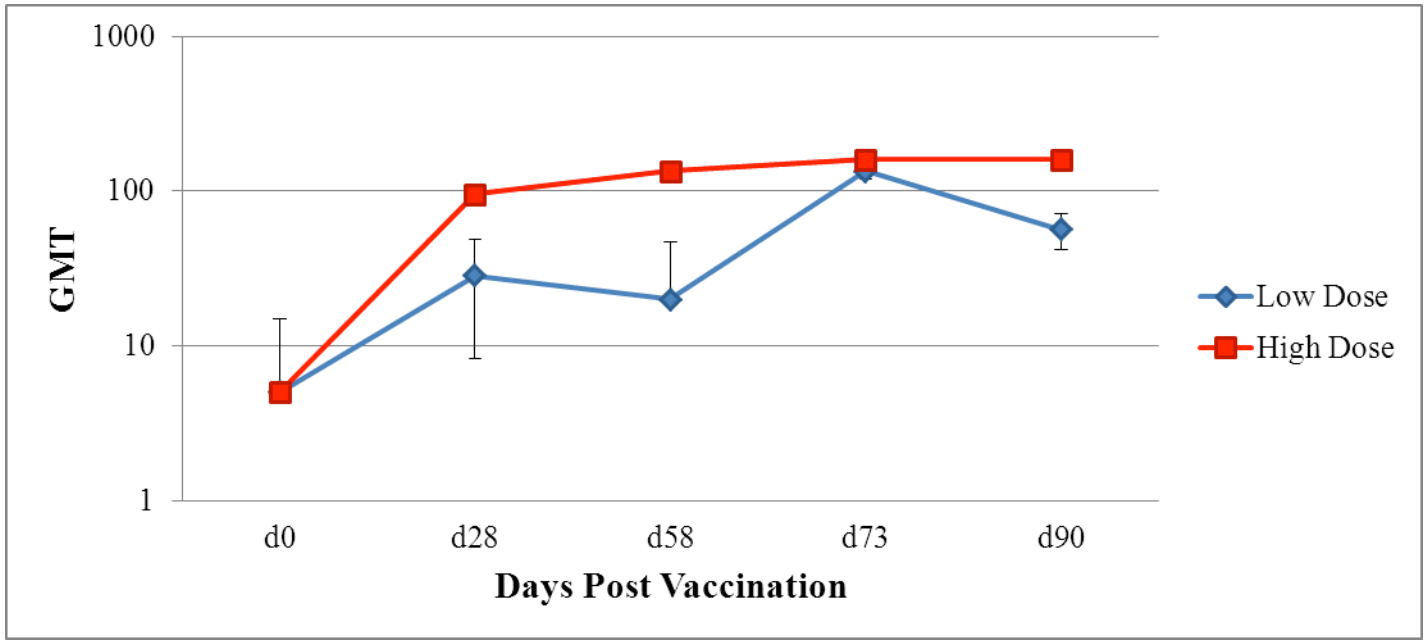

Figure 2.6 **Immunogenicity of high dose and low dose DENVax in non-human primates.** High dose tetravalent DENVax contains one log higher (10^6 PFU) DENVax-4 than low dose DENVax (10^5 PFU DENVax-4). DENVax-4 1st generation was used in this experiment. GMT values are measurements of neutralizing antibody titers from plaque reduction neutralization test with DENV-4. High dose values (red) and low dose values (blue) were measured at four time points post vaccination.

2.4 DISCUSSION

One of the frequent issues with developing a multivalent vaccine is ensuring that the vaccine provides significant immunogenicity against all targeted virus strains or stereotypes. This is often termed a "balanced immune response." DENVax contains 4 vaccine strains to protect against the four stereotypes of DENV that cause dengue fever. Three of the four stereotypes elicit a significant neutralizing antibody response in mice and provide protection upon challenge with wild type dengue virus. Data for DENVax-4 show a much lower immune response in mice. This suggests that the neutralizing antibody titers are much lower than those produced by the other DENVax strains, resulting in possible reduced seroconversion and protection from infection. Previous attempts to reengineer the DENVax-4 clone showed no increase in Vero cell growth kinetics. In this study we successfully performed blind serial passages on both the DENVax-4 first generation clone and the DENVax-4b second generation clone. We also performed a growth kinetics analysis to observe any differences in peak titer between the passages. Finally, we completed sequencing of genomes of the first and tenth passages of each construct to note any mutations in the genomic RNA sequence.

Both DENVax-4 and DENVax-4b were blindly passaged 10 times in Vero cells. During passages CPE was noted weekly on each cell flask. There were no significant differences in CPE noted, indicating sufficient attenuation in both the DENVax-4 and DENVax-4b passages. Samples from each flask were taken before subsequent passaging, and all samples were titrated at the conclusion of the serial passages. There was no significant difference in peak titer between the two viruses after passaging, and both had similar peak titer increases over the course of the 10 weeks. This warranted further testing in Vero cells to determine growth kinetics because the

DENVax-4b strain had reached similar titers to that of DENVax-4, showing potential for an increased initial growth rate *in vitro*.

A growth kinetics analysis in Vero cells was completed with samples of DENVax-4, DENVax-2, DENVax-4-P10 and DENVax-4b-P10. All four samples produced significant infectious titers in Vero cells. DENVax-2 reached the highest peak titer on day 4, and had the highest titer on day 12. DENVax-4b-P10 reached the highest peak titer of the DENVax-4 clones tested, and also had the highest titer of the DENVax-4 clones on day 12. DENVax-4b-P10 also reached a higher titer on day 12 than DENVax-4. However, the differences between the titers of the DENVax-4 clones were not statistically significant. DENVax-4b did produce peak titers similar to that of DENVax-4, which warranted testing in mice and non-human primates to determine if there is an increase in vaccine efficacy against DENV-4 between the two vaccine strains, since immunogenic protein structure also plays a role in immunogenicity. If DENVax-4b produced immunogenic proteins in the host that generated stronger neutralizing antibodies, the immunogenicity would increase compared to DENVax-4.

Vaccine doses containing either DENVax-4 first generation or DENVax-4b-P10 were formulated and used to immunize AG 129 Mice and NHP. The neutralizing antibodies produced in the mice were not significantly different from the control values. No increase in antibody titer was noted in mice vaccinated with either DENVax-4b-P1 or DENVax-4b-P10. This suggests that the strain's adaptations did not result in increased immunogenicity.

In the NHP study, neutralizing antibody responses were measured from each of the five groups to compare immunogenicity and test the effect of vaccine dose schedule, DENVax-4b-P10 response versus DENVax-4 response, and increased DENVax-4 dose. The results of the NHP study suggested that second generation DENVax-4b-P10 did not result in improvement compared to DENV-4 neutralizing antibody responses. Increased DENVax-4 in the dose formulation did show a significant increase in neutralizing antibody production. Overall the results suggest that further modification of DENVax-4 was needed to improve immunogenicity, as the antibody response to DENV-4 did not improve with the new constructs.

A successful DENV-4 vaccine should be able to elicit a response that adequately neutralizes multiple strains of wild type DENV-4 including newly evolved strains with genome modifications, different genotypes and different phenotypes. To date no studies have been done to test whether DENVax4 establishes an immune response against other DENV-4 strains. A future experiment with sera from this study will be to test neutralizing antibody response against multiple strains of DENV-4.

The results described above indicate that increasing Vero cell adaptation of DENVax-4 by blind serial passaging was not effective in improving growth kinetics in Vero cells, specifically because there was no difference in peak titer between either DENVax-4-P1 and P10 or DENVax-4b-P1 and P10. During genome sequencing there were three point mutations identified between passage 1 and passage 10 in both constructs. These mutations were located in the capsid region of DENVax-4b and in the prM and envelope genes of DENVax-4. Individually incorporating these mutations into the DENVax-4 or 4b backbones may provide increased growth in Vero cells

that was not conferred by all three together. These mutations may also decrease the attenuation of the virus, which could improve immunogenicity in mice. A future experiment is to clone these mutations into the DENVax-4 or DENVax-4b construct, transcribe RNA and rescue live virus, and test for improved growth kinetics in Vero cells and immunogenicity in mice.

CHAPTER 3

GENERATION AND CHARACTERIZATION OF MODIFIED DENVAX-4 VACCINE VIRUSES

3.1 BACKGROUND AND RATIONALE

The use of live virus to vaccinate an individual is now a common practice in medicine. Vaccines containing live virus provide stronger long-term immunity against pathogens due to replication of virus within the host. To ensure the vaccinee develops no clinical symptoms of disease, the vaccine virus is significantly attenuated. The attenuating phenotypes of DENVax are reduced replication, temperature sensitivity, reduced neurovirulence, reduced plaque size and reduced ability for growth in mosquito cells to prevent vaccine virus transmission. Inviragen's current DENVax-4 strain used in DENVax contains prM and E genes from the wild type DENV-4 strain 1036. During construction of DENVax-4 three clones were made. D2/4-P contains the prM and E genes from 1036 in a backbone of wild type DENV-2 16681. D2/4-E contains prM and E genes from 1036 in a backbone of PDK-53 E variant, which has eight of the nine attenuating mutations in the PDK-53-V backbone. D2/4-V is the current DENVax4 clone, containing prM and E genes from 1036 in the PDK-53-V backbone. D2/4-P showed significant immunogenicity in AG129 mice after both primary immunization and boost, but also showed a 62.5 percent mortality rate, demonstrating that this strain is under-attenuated for use as a vaccine. D2/4-E also showed a significant increase in immunogenicity compared to D2/4-V after boost, and no

mortality resulted. However, D2/4-V elicited decreased neutralizing antibody titers and decreased immunogenicity in AG129 mice (Figure 3.1). This suggests that reverting select attenuating mutations in the PDK-53-V backbone to wild type DENV-2 sequence may increase the immunogenicity of DENVax-4 in mice.

Of the nine attenuating mutations in the DENVax-2-PDK-53 backbone, three are silent mutations. It is understood that the three main attenuating mutations reside in the 5'UTR, NS1 and NS3 (20). However, since the pD2/4-P and E strains showed higher immunogenicity in AG129 mice, reversion of one or more of these mutations may have an effect on DENVax-4 fitness and thus boost its immunogenicity *in vivo*. The hypothesis for this project is that reversion of noncritical attenuating mutations in the 5' NCR, NS2A and NS4A genes to the wild-type DENV-2 16681 sequence will increase immunogenicity of the vaccine strain in mice. Three candidate vaccine constructs have been generated based on this hypothesis. DENVax-4f contains reversion of the attenuating mutations in the NS2A and NS4A genes. DENVax-4g contains reversion of the attenuating mutations at 5' NCR, NS2A and NS4A. DENVax-4j contains a reversion at the 5' NCR. These three constructs were created by Claire Huang and Richard Kinney at the CDC in Fort Collins during the original cloning of DENVax-4. Since stocks were readily available the CDC generously donated the clones for testing at Inviragen.

TABLE 6. Immunogenicity of D2/3 and D2/4 chimeras in AG129 mice[a]

Virus	PRNT titer[b]	
	Primary	Boosted
D3 16562	160 (320)	320 (640)
D2/3-P	160 (320)	320 (640)
D2/3-E	40 (160)	160 (640)
D2/3-V	80 (160)	320 (640)
D4 1036	320 (640)	640 (1,280)
D2/4-P	160 (320)	320 (640)
D2/4-E	20 (80)	160 (320)
D2/4-V	20 (40)	80 (320)

[a] Six- to eight-week-old AG129 mice were immunized with 10^5 PFU of virus, bled on day 40, and boosted with the same dose of virus on day 42. Mice were bled again 26 days after boosting.

[b] Reciprocal dilution of pooled sera in each group yielding at least 70% (or 50%, in parentheses) plaque reduction of the D3 16562 (for D3 and D2/3 groups) or D4 1036 (for D4 and D2/4 groups) virus.

Figure 3.1 **Immunogenicity of DENVax-3 and DENVax-4 chimeras in mice.** D2/4-P and D2/4-E showed high neutralizing antibody titers after primary and secondary immunizations when compared to D2/4-V (DENVax-4).

Huang C Y-H et al. (2003) Dengue 2 PDK-53 Virus as a Chimeric Carrier for Tetravalent Dengue Vaccine Development. *Journal of Virology* 77: 11436-11447.

In addition to attenuating mutation reversions, new DENVax-4 constructs were designed that contained mutations found by serial passages of DENVax-4 first generation and DENVax-4b second generation in Vero cells (Figure 3.2). Blind serial passaging of viruses is a method used to select for increased in vitro growth rates, and increased growth rate may contribute to increased immunogenicity in mice. DENVax-4 and DENVax-4b were both blindly passaged 10 times in Vero cells. Following passaging the genomes of the first and tenth passages of each virus was sequenced to compare viral genomes. DENVax-4b had one non-synonymous mutation in the capsid protein at position 107 which changed a cysteine to a tyrosine. DENVax-4 had two non-synonymous mutations, one in the envelope protein (position 417, glutamate to lysine) and one in the NS4A protein (position 17, methionine to leucine). Three new vaccine constructs were designed and created based on these sequencing results. DENVax-4e contains the capsid cysteine to tyrosine mutation in the DENVax-4b backbone. DENVax-4h contains the envelope 417 glutamate to lysine mutation in the DENVax-4 backbone. DENVax-4i contains the NS4A 17 methionine to leucine mutation in the DENVax-4 backbone.

DENVax-4e, DENVax-4h, and DENVax-4i were cloned and amplified in Vero cells at Inviragen, and experiments done with these constructs were completed in conjunction with experiments done on DENVax-4f, DENVax-4g, and DENVax-4j provided by the CDC. All constructs were analyzed in vitro by performing growth curves in Vero cells to test for increased growth rate, and in C6/36 cells to test for decreased replication in cultured mosquito cells. Based on data from the growth curve analysis 3 constructs were selected and tested in AG129 mice to compare *in vivo* immunogenicity with DENVax-4.

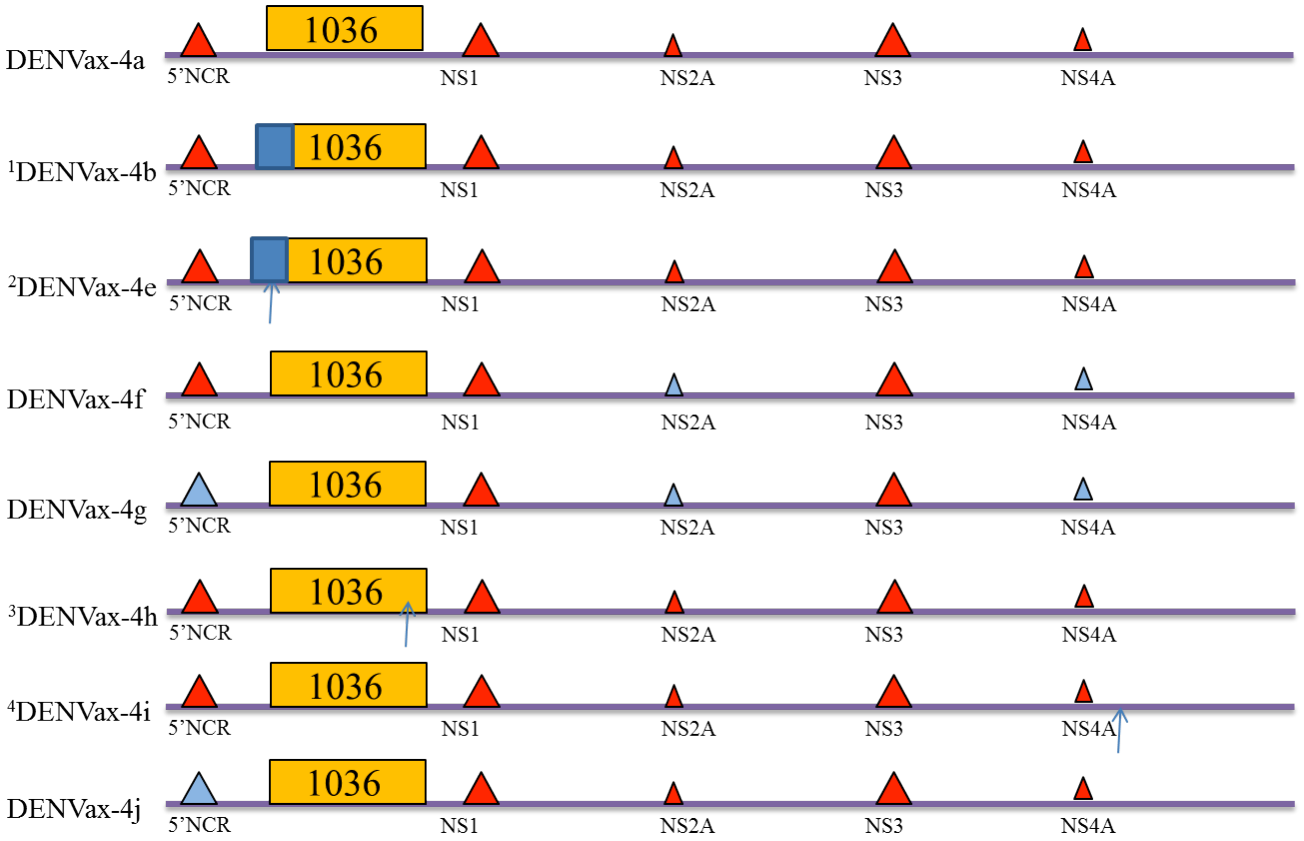

Figure 3.2 **Schematic of DENVax-4 second generation constructs.** Red triangles indicate attenuating mutations in the DENVax-2 PDK-53 backbone. Blue triangles in the DENVax-4f, DENVax-4g, and DENVax-4j constructs indicate reversions to wild type sequence at the attenuating mutations.

[1] DENVax-4b contains modifications at the capsid/prM junction (blue).

[2] DENVax-4e contains a C to Y mutation at capsid 107 (arrow), and has a DENVax-4b backbone.

[3] DENVax-4h contains an E to K mutation at envelope 417, and has a DENVax-4 backbone.

[4] DENVax-4i contains an M to L mutation at NS4A 75, and has a DENVax-4 backbone.

3.2 MATERIALS AND METHODS

3.2.1 CLONING OF DENVAX-4E

DENVax-4e was created using site-specific mutagenesis and molecular cloning techniques. Forward and reverse primers were designed using a sequence containing the necessary nucleotide mutation 3' of the AgeI restriction site (Table 3.1). These primers amplified an insert flanked by the AgeI and MluI restriction sites in a PCR reaction. The template used was a pUC57 plasmid containing the region of DENVax4b plasmid between the AgeI and MluI sites (Figure 3.3). This construct was an intermediate from the previous DENVax4 modification study. The insert was digested with AgeI and MluI for 1 hour at 37ºC and purified using the GENECLEAN DNA purification kit (MP Biomedicals, Solon, OH). The ligation used T4 DNA ligase with 1:4 insert to vector ratio. The reaction was carried out at room temperature for 10 minutes. The transformation used 2 µL of the ligation reaction and 50 µl XL-1 blue competent cells thawed on ice. Cells were incubated on ice with 2 µl of the ligation reaction for 30 minutes, heat shocked in a 42ºC water bath for 30 seconds, and placed on ice for 2 minutes. After transformation 450 µl 2xYT medium was added to the cells. Cells were then grown on a shaker incubator at 37ºC for 1 hour. Cells were then spread on 2xYT agarose plates containing ampicillin using glass beads and incubated overnight at 37ºC. Colonies were picked into 200 mL 2xYT plus ampicillin medium the next morning and grown overnight to amplify cultures. Plasmids from large scale cultures were purified using a QIAfilter midi-prep kit (Qiagen, Hilden, Germany).The insert in the pUC57 intermediate was sequenced, and then removed using an AgeI/MluI digest. The insert was then ligated into the digested pD2/4b backbone, and amplified in XL1-blue competent cells.

Table 3.1 **Primers used in cloning of DENVax-4e and mutagenesis of DENVax-4h and DENVax-4i**. Forward primer sequences are given 5' to 3'. Reverse primer sequences are given 3' to 5'.

	pD2/4-e	pD2/4-h	pD2/4-i
Forward Primer	5'gctcaccggtaaatttaatacga	5'cgaatggccattctaggtaaaacagcttgggattttg	5' taggctcccaaccttcttgactcagaaggcaag
Reverse Primer	3'atcacgcgttgacaagtgaaacgccat tacggtgggaatcaagtacagcaatgttat cgttgagct	3'caaaatcccaagctgttttacctagaatggccattcg	3' cttgccttctgagtcaagaaggttgggagccta

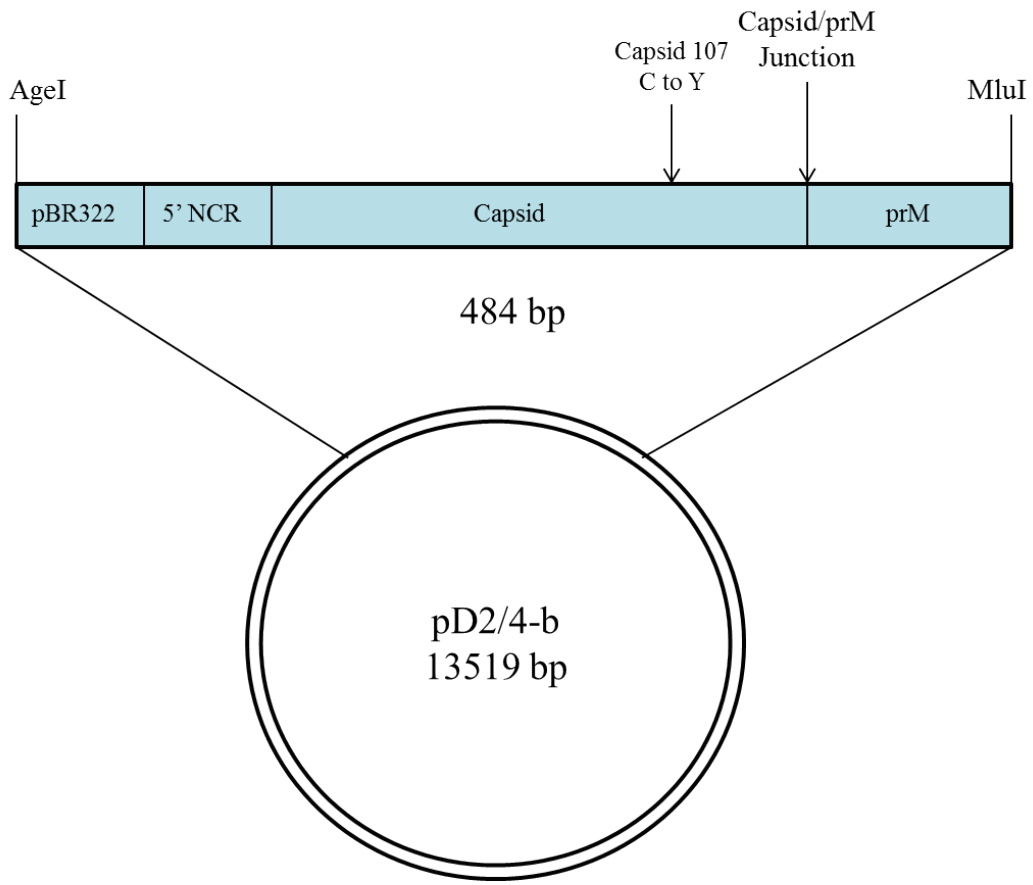

Figure 3.3 **Model of the modification to pD2/4-b to generate pD2/4-e.** The reverse primer flanking the MluI site contained a nucleotide G to A change at position 416 to change the amino acid residue at capsid 107 to a tyrosine.

3.2.2 PLASMID MUTAGENESIS CLONING OF DENVAX4H AND DENVAX4I

DENVax-4h and DENVax-4i were generated using mutagenesis reactions in a QuikChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). Forward and reverse primers were designed that contained the desired mutation and flanked the same unmodified sequence on the pD2/4 template (Table 3.1). The kit contains a DNA polymerase that uses the primers to amplify the entire plasmid which contains the nucleotide mutation encoded by the primer (Figure 3.4). The PCR amplification reaction of the new plasmids contained 10x reaction buffer, 10 ng of the unlinearized DNA plasmid template, 125 ng of each primer, dNTP mix, and quik solution reagent to enhance amplification efficiency. The PCR consisted of 3 steps; 95ºC for 50 seconds, 60ºC for 50 seconds, and 68ºC for 13 minutes (1 minute/ kb of plasmid). These steps were repeated for 18 cycles, followed by a single elongation step at 68ºC for 7 minutes. After PCR amplification DpnI enzyme was added to each reaction to digest the template plasmid. The mutated plasmid was not digested because DpnI enzyme targets methylated DNA found on the template plasmid but not the mutated plasmid. The reaction was digested for 1 hour at 37ºC. During this time XL-10 gold super competent cells were thawed on ice, and prepared for transforming by supplementation with beta-ME solution to improve transformation efficiency and stability of the plasmid. Beta-ME solution was added to the competent cells and incubated for 10 minutes. After DpnI digestion was completed 2 µl of each reaction was added to 45 µl of XL-10 gold cells and tapped to mix. Transformation reactions were incubated on ice for 30 minutes, heat shocked in a 42ºC water bath for 30 seconds, and incubated on ice for 2 minutes. During the incubations NZY+ medium was prewarmed to 42ºC and 500 µl was added to each reaction after 2 minutes on ice. The template plasmid was

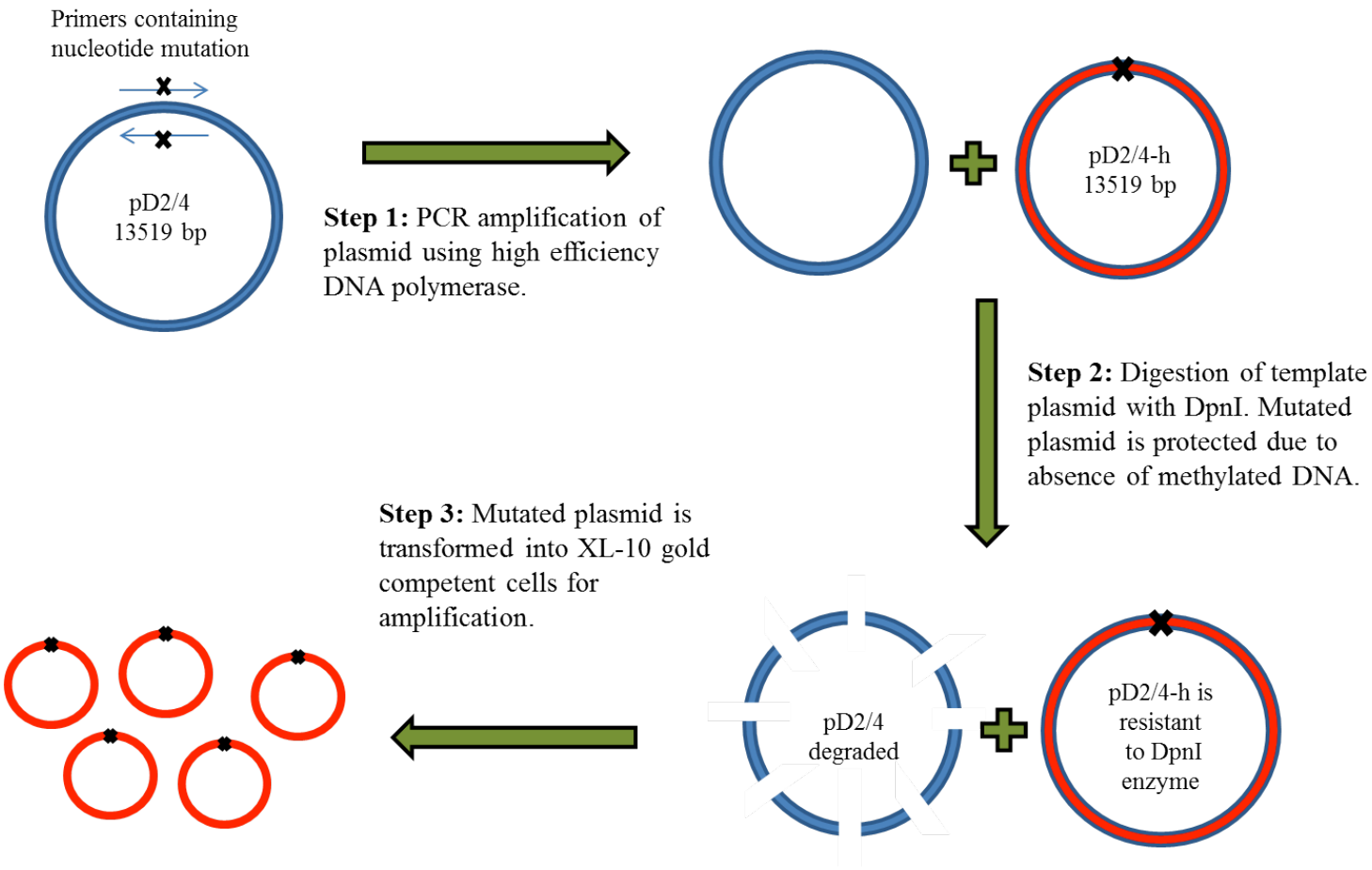

Figure 3.4 **Model depicting the cloning method for DENVax-4h and DENVax-4i.** The DNA polymerase (*PfuUltra* high-fidelity) used to amplify the infectious clones has an 18-fold higher fidelity than Taq DNA polymerase. Plasmids are amplified in Xl-10 gold ultra-competent E. coli cells, which have a 5-fold increase in transformation efficiency than XL1-blue cells, as well as an Hte phenotype that increases the transformation efficiency of larger DNA plasmids. Protocol and kit were obtained from Agilent Technologies.

digested by DpnI and then the mutated plasmid was transformed into XL10-gold super competent cells, which have been optimized to grow unstable plasmids. A pWhitescript control plasmid was used to determine the success of the mutagenesis reaction.

3.2.3 SEQUENCING

Full sequencing of the DENVax-4h and DENVax-4i plasmids was necessary as a result of completely new plasmid construction, and was completed after amplification. Primers were designed using previous records from scientists at the CDC on DENVax-4 plasmid sequencing, and ordered from Life Technologies. Sequencing reactions were performed by Beckman Coulter Genomics. Sequencing of the modified insert in the DENVax-4e plasmid was also completed by Beckman Coulter Genomics. Chromatograms were analyzed using Finch TV (Geospiza Inc., Seattle, WA) and CLC Sequence Viewer Software (CLC bio, Aarhus, Denmark). Full scale plasmid sequencing was not necessary because the DENVax-4e plasmid backbone is from the previously sequenced DENVax-4b plasmid.

3.2.4 RNA TRANSCRIPTION

RNA was transcribed from each of the three plasmids using an ampliscribe kit (Epicentre Technologies, Madison, WI). The plasmid DNA templates for each construct were linearized by

XbaI digestion and purified using a GENECLEAN DNA purification kit. To promote efficient capping of the mRNA, the concentration of ATP in the dNTP mix was reduced by 1/5, and 200 ng of digested template DNA was used. The reaction was performed at 37ºC for 2 hours, and then placed on ice before electroporation.

3.2.5 CELL CULTURE

Vero cells are mammalian cells derived from African green monkey kidney. The Vero cell line used in the in vitro experiments was obtained from the Waisman Cell Bank (Waisman Manufacturing, Madison, WI). Vero cells were grown at 37ºC in Dulbecco's Modification of Eagle's Medium (DMEM, Mediatech Inc., Manassas VA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan UT), 2% L-glutamine (Hyclone), and 1% penicillin-streptomycin (Pen-Strep, Hyclone). To passage the cells Tryple Express solution (Life Technologies, Grand Island NY) was used to remove the cells from the flask surface. Cells were diluted 1:24 for passage once a week, and passaged 20 times before new cells were thawed.

C6/36 cells are *Aedes albopictus* mosquito cells that were obtained from stocks at the CDC and grown in DMEM supplemented with 10% FBS, 2% L-glutamine, 1% pen-strep, 1% Non-essential amino acid solution (Life Technologies, Carlsbad, CA), and 1% sodium pyruvate solution (Mediatech Inc., Manassas, VA). Cells were grown at 28ºC, and scraped off flask surface for passaging using rubber cell scraper, and diluted 1:24 for passaging every week. After 20 passages new cells were thawed.

3.2.6 ELECTROPORATION

After RNA transcription the reaction mix was transferred to an electroporation cuvette containing 400 µl Vero cells at $1x10^7$ cells/mL. A BTX ECM 830 electroporater from Harvard Apparatus was used. The electric pulse was 225V for 25 msec. Cells were then transferred to an eppendorf tube and incubated at room temperature for 10 minutes. The mix was then transferred to a flask containing DMEM 5% FBS and incubated at 37ºC overnight. Medium was changed after 24 hours to remove cell debris.

3.2.7 VIRUS AMPLIFICATION

Virus was grown in flasks containing the electroporated cells for 12 days. The virus-containing media was harvested on day 12 and stored at -80°C. Two mL of each virus stock was adsorbed to a new Vero monolayer on a T-75 flask for 60 minutes with rocking to prevent drying of the cells. After adsorption 10 mL of DMEM 2% FBS was added to each flask. Flasks were incubated for 12 days and the medium was harvested and stored at -80C. This generates the passage 2 (P2) stocks used for subsequent testing. Infectious viruses were titrated by immunofocus assay.

3.2.8 *IN VITRO* GROWTH KINETICS

Growth curves for each of the new DENVax-4 constructs were done in Vero cells to assess peak titer in comparison to DENVax-4. Vero T-75 flasks were seeded 2 days prior to infection. Flasks were infected at a MOI of 0.001 with P2 of each of the DENVax constructs. DENVax-4, DENVax-2, and 1036 wild type DENV-4 were used as controls. Growth medium, DMEM without FBS, was changed on days 2, 4, and 6-12. Flasks were washed 2 times with PBS during medium changes. Viruses were grown for 12 days, and samples were taken on days 2 and 4-12 for plaque titration by IFA. Growth curves for each construct including the controls were done in duplicate; two flasks per construct.

Growth curves were also conducted using C6/36 cells to test for decreased replication and increased attenuation when compared to wild type DENV-4 1036 and DENVax-4. C6/36 T-75 flasks were seeded 2 days prior to infection. Flasks were infected at a MOI of 0.001 with P2 of each of the DENVax constructs. DENVax4, DENVax2, and 1036 wild type dengue 4 were used as controls. Growth medium was DMEM without FBS, and was changed on days 2, 4, and 6-12. Flasks were washed 2 times with PBS during media changes. Viruses were grown for 12 days, and samples were taken on days 2 and 4-12 for plaque titration by IFA. Growth curves for each construct including the controls were done in duplicate; two flasks per construct.

3.2.9 IMMUNOFOCUS ASSAY

To assess virus titer, growth curve samples were analyzed by an immunofocus assay instead of a traditional plaque titration done in previous studies. Several issues arose with identifying plaques using neutral red solution because the clarity depends heavily on the quality of the batch of neutral red used. The immunofocus assay is an antigen staining technique allowing for visualization of virus-infected cell foci on the Vero monolayer. Virus samples were adsorbed to Vero monolayers pre seeded on 6-well plates for 90 minutes with rocking every 8 minutes. After adsorption wells were over-laid with a 1:1 solution of 1.4% carboxymethyl cellulose (CMC) and 2x DMEM containing 2% FBS. Plates were incubated for 7 days, and then the wells were washed 3 times with PBS to remove the CMC. Cells were fixed using 1 mL per well of cold methanol and incubated at -20ºC for approximately 20 minutes in Ziploc bags. After fixing the methanol was removed and plates were air dried in a chemical fume hood for approximately 40 minutes. Wells were then washed with PBS. The primary antibody was a mouse monoclonal antibody against DENV-4whose target has not been mapped. The stock antibody was diluted 1:2000 in a blocking buffer solution containing 2.5% dry milk and 0.5% tween-20 in PBS. Primary solution was added at 1 mL per well. Plates were incubated overnight at 4ºC. Primary antibody was then removed and wells washed 2 times with wash buffer. Secondary antibody was anti-mouse serum from goats conjugated with horse radish peroxidase (HRP) (Southern Biotechnologies, Birmingham, AL). The secondary antibody was diluted 1:1000 in blocking buffer and added 0.5 mL per well. Plates were incubated at 37ºC for 1 hour. Wells were washed 2 times with wash buffer and 350 µl per well of substrate was added. Plates were incubated at room temperature until foci were visible, approximately 30 minutes. Wells were then washed once with distilled H_2O to stop the reaction. Plates were inverted and left to dry for 24 hours,

after which foci were counted. Titers were calculated by multiplying the number of foci in the well by the dilution factor.

3.2.10 AG129 MOUSE STUDY

To test the efficacy of the second generation DENVax-4 constructs the AG129 mouse model was used. AG 129 mice are deficient in interferon alpha, beta, and gamma receptors (58). As a result interferon, which has an important role in the DENV immune response in humans, cannot affect the immune response in mice. This makes AG129 mice a good small animal model for DENV vaccine development because they show clinical symptoms when infected AG129 mice were vaccinated with either DENVax-4 1st generation, DENVax-4e, DENVax-4f, DENVax-4h, or PBS control. Six animals approximately 4 weeks of age were used in each group. Mice received primary vaccinations on day 0 and bled on day 0, day 42, and day 56 to measure neutralizing antibodies. On day 56 mice were challenged with wild type DENV-2. Mice were bled on day 3 and day 59 to test for viremia post-immunization and post-challenge. Morbidity and mortality were monitored for the duration of the study. The mice were vaccinated and challenged at Inviragen in Madison, WI. Serology analysis was completed at Inviragen in Fort Collins, CO.

3.2.11 MICRONEUTRALIZATION ANALYSIS

To measure neutralizing antibody response in mouse sera, a microneutralization assay was used. On day -1 Vero cells were seeded on 96-well plates for day 0 confluence. On day -1 sera/virus

dilutions were also prepared for overnight incubation. Sera samples were diluted 2-fold in DMEM supplemented with 2% FBS, 2% L-glutamine, and 1% Pen-strep. The starting dilution of mouse sera was 1:20. After dilutions were completed approximately 240 PFU of DENV-4 1036 were added per well. Dilution plates were incubated overnight at 4ºC for approximately 18 hours. On day 0 medium was aspirated off Vero 96-well plates and 30µl of a dilution was added to each well. Virus/sera dilutions were adsorbed to the monolayers for 90 minutes, then wells were overlaid with a 1:1 ratio of 1.4% methyl cellulose and 2x DMEM supplemented with 4% FBS. Plates were incubated at 34ºC for 48 hours. After virus replication methyl cellulose overlay was removed and wells were washed once with PBS. Monolayers were then fixed with cold methanol and incubated at -20ºC for approximately 90 minutes. After fixing methanol was removed and plates were air dried. Plates were then washed 1x with wash buffer. Primary antibody diluted 1:1000 in milk blocking solution was then added at 50 µl per well. Plates were incubated overnight at 4ºC for approximately 18 hours. Primary antibody was then washed off the wells using wash buffer. Secondary antibody was diluted 1:200 in milk block solution and was added at 50 µl per well. Plates were incubated at 37ºC for approximately 1 hour. During this time the substrate was prepared by dissolving 1 3-amino-9-ethlycarbazole (AEC) tablet in 2.5 ml of dimethyl sulfoxide (DMSO). Once dissolved 47.5 ml of 50 mM acetate buffer was added to make 50 ml of substrate. After incubation secondary antibody was washed off the plates using wash buffer. To complete the substrate solution 250 µl 3% hydrogen peroxide was added. The solution was mixed and then 50 µl per well was added. Plates were incubated at room temperature for 20 minutes to ensure full development of red foci. Plates were then washed with tap water and dried overnight. Foci were counted using an Elispot plate reader (Autoimmun Diagnostika, Strassberg, Germany).

3.2.12 PLAQUE SIZE ANALYSIS

To test for attenuation in vitro, measurements of viral plaques were taken and compared to wild type DENV-4 1036 plaques. On day -2, Vero cells were seeded on 6-well plates to ensure confluence. On day 0 Viruses were diluted in serial 10-fold dilutions in DMEM supplemented with 2% FBS, 1% L-glutamine, and 1% Pen-Strep. Medium was aspirated off the Vero cell wells and a 100 μL sample of virus dilution was added. Virus was adsorbed to the monolayers for 90 minutes with rocking every 8 minutes to prevent drying. After adsorption wells were overlaid with 4 mL per well 1:1 solution of 1.6% agarose gel and BSS (NaCl, KCl, NaH_2PO_4-H_2O, glucose, $CaCl_2$-$2H_2O$, $MgSO_4$-$7H_2O$). Plates were incubated at 37ºC for 4 days, then overlaid with 2 mL per well of 1:1 BSS/1.6% agarose solution supplemented with neutral red. Plaques were measured on days 5, 6, and 7. Ten plaques were measured for each construct and the diameters were averaged. A student t-test was done to determine the significance of the size differences compared to DENV-4 1036.

3.3 RESULTS

3.3.1 GENERATION OF DENVAX-4E PLASMID

The DENVax-4e plasmid was made using a fragment from DENVax-4b. The fragment was flanked by the AgeI and MluI restriction enzyme sites, and was purified by gel electrophoresis

after digesting the DENVax-4b plasmid with AgeI and MluI. The DNA fragment was cut from the gel and cleaned using the GENECLEAN kit. After purification the fragment was used as a PCR template and amplified using a primer that contained the desired nucleotide mutation. After PCR amplification the insert contained the mutated sequence that introduces a C-Y mutation at amino acid 107 in the capsid sequence. This insert was ligated into a pUC-57 intermediate and amplified in DH5- alpha *E. coli* competent cells. Colonies picked were grown in LB/AMP media overnight and then plasmids were purified using a Qiagen Mini-Prep kit. The insert region of the plasmid was sequenced to confirm that the desired mutation had been introduced into the insert that had been amplified. The intermediate plasmids and the DENVax-4b full length plasmid were digested with AgeI and MluI. The DENVax-4b vector and insert from intermediate plasmid were purified by gel electrophoresis and cleaned using the GENECLEAN kit. The insert was then ligated into the vector and transformed into XL-1 blue *E. coli* competent cells. Colonies grown on 2xYT plates were amplified in 2xYT/AMP media and plasmids were purified using a Qiagen Midi-Prep kit. The full length plasmid was sequenced upon purification.

3.3.2 GENERATION OF DENVAX-4H AND DENVAX-4I PLASMIDS

Infectious cDNA clone bacterial DENVax plasmids are large and very unstable when transformed into competent cells. It is common for large deletions to occur in the plasmid due to the difficulty to replicate the plasmid. IC DENVax plasmids also result in reduced cell viability. As a result the only colonies will either be small with full length IC plasmids or healthy with

plasmids containing deletions. Previous DENVax cloning used XL-1 blue competent *E. coli* cells because of their known stability with large plasmids. Traditional cloning methods as described above were first used in the DENVax-4h and DENVax-4i cloning, but transformation attempts in XL-1 blue cells produced no colonies or plasmids with deletions. A new cloning procedure was developed that involved the use of a plasmid mutagenesis kit purchased from Agilent Technologies. A high efficiency DNA polymerase was used to generate full length IC DENVax-4 plasmids using two primers containing the desired nucleotide mutation for each construct. This encoded the desired nucleotide mutations to make DENVax-4h or DENVax-4i. Upon synthesis the template plasmid was degraded using DpnI enzyme. The plasmids were transformed into XL-10 gold *E. coli* competent cells. These cells are designed to allow for replication of large unstable plasmids. The colonies were grown on LB/AMP plates and picked into LB/AMP media for overnight amplification. Because of the high replication efficiency of these cells only 3 mL cultures were grown. Plasmids were purified using a Qiagen Mini-Prep kit. The full length plasmid for each construct was sequenced.

3.3.3 VIRUS RESCUE AND AMPLIFICATION

Each full length plasmid was digested with XbaI to linearize the DNA, which was then transcribed into full length genomic RNA. After RNA transcription the RNA was electroporated into Vero cells for virus production. Virus was grown on Vero cells for 12 days before harvest. This yielded passage 1 of each new construct (DENVax-4e, DENVax-4h, and DENVax-4i).

Vero cells seeded 2 days prior to passage 1 harvest were infected with passage 1 samples of each virus to generate passage 2. DENVax-4e-P2 was harvested 5 days after infection because the monolayer was showing severe cell detachment. DENVax-4h-P2 and DENVax-4i-P2 were harvested 7 days after infection for the same reasons.

3.3.4 PHENOTYPIC CHARACTERIZATION IN VERO CELLS

A growth curve analysis in Vero cells was done for each of the modified DENVax-4 clones to compare growth efficiency and peak titer to DENVax-4 first generation (Figure 3.5). Controls used in the growth curves were DENVax-4 and DENVax-2. Each construct was grown in duplicate flasks for data comparison. Wild type 1036 virus reached the highest peak titer on day 6. DENVax-4e reached the highest peak titer of the DENVax-4 constructs on day 6. DENVax-4i reached the lowest peak titer of the DENVax-4 constructs on day 6. All viruses reached a similar titer on day 12, with the exception of wild type 1036 (Figure 3.5).

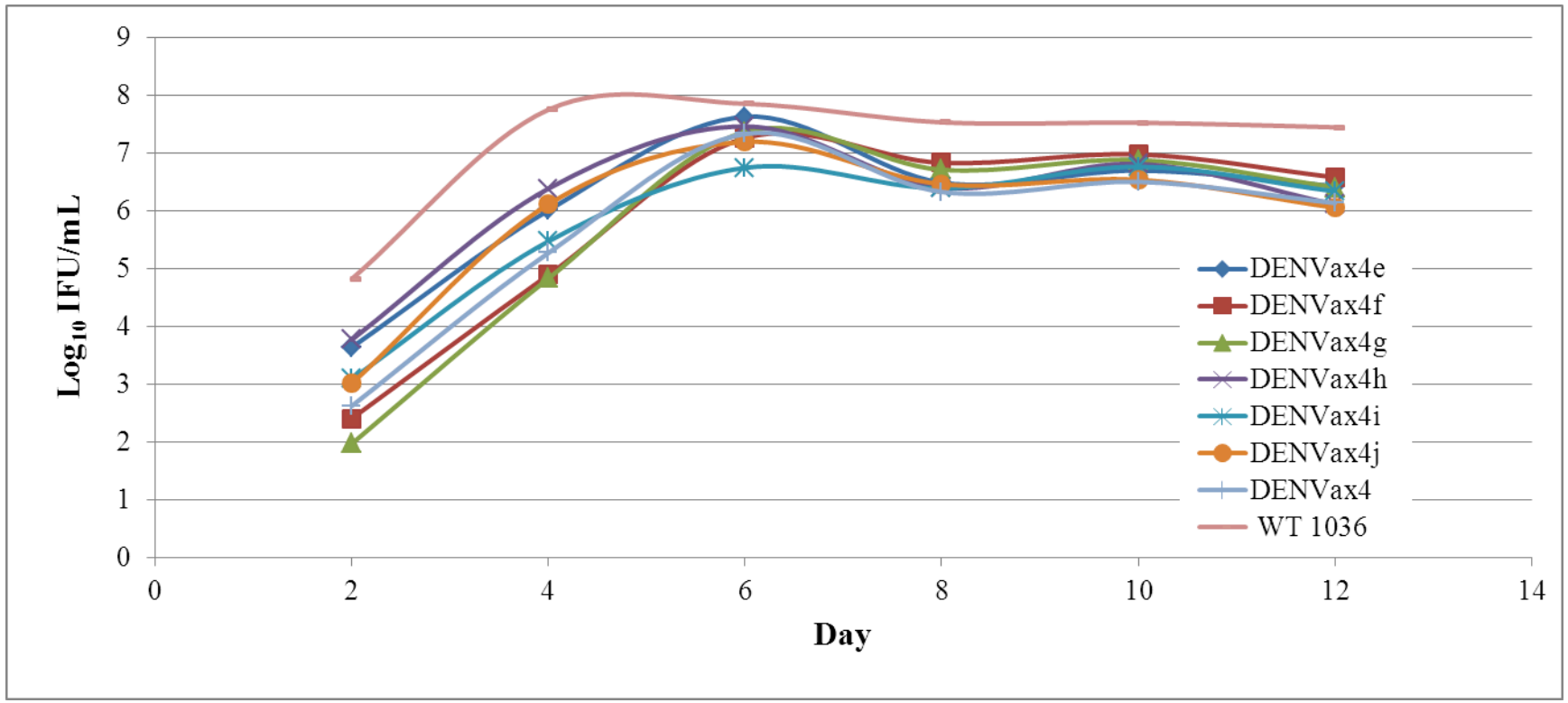

Figure 3.5 **Growth Kinetics of DENVax-4 constructs in Vero cells.** Constructs were grown on Vero cells for 12 days. Samples taken on designated days were titrated by immunofocus assay.

3.3.5 ATTENUATION CHARACTERIZATION IN C6/36 CELLS

A growth curve analysis was done in C6/36 cells to test for decreased replication in mosquito cells (Figure 3.6). Wild type DENV-4 1036 reached the highest peak titer on day12. DENVax-4 reached the highest peak titer of the DENVax-4 constructs. DENVax-4f reached a significantly lower peak titer than the other DENVax-4 constructs, and did not show detectable virus growth until day 8. DENVax-4e, DENVax-4, 1036, DENVax-4i, and DENVax-4j showed virus production at day 2. DENVax-4g and DENVax-4h showed virus production on day 4. All constructs showed increasing titers through day 12.

3.3.6 IMMUNOGENICITY IN AG129 MICE

Based on the growth kinetics analysis done in Vero and C6/36 cells, 3 constructs were tested against DENVax-4 for an increase in immunogenicity in mice. DENVax-4e and DENVax-4h were selected based on their high peak titers in Vero cells. DENVax-4f was selected because of the significant decreased peak titer in C6/36 cells (Table 3.2). Mice were immunized with a 10^5 PFU/mL dose on day 0. Serology was done on samples taken day 45, day 56, and day 71 using a microneutralization assay. To interpret the data geometric mean titer (GMT) values were analyzed. These values indicate the dilution of mouse serum containing sufficient antibodies to effectively neutralize 50% of the added virus. To determine these dilutions, a back titration

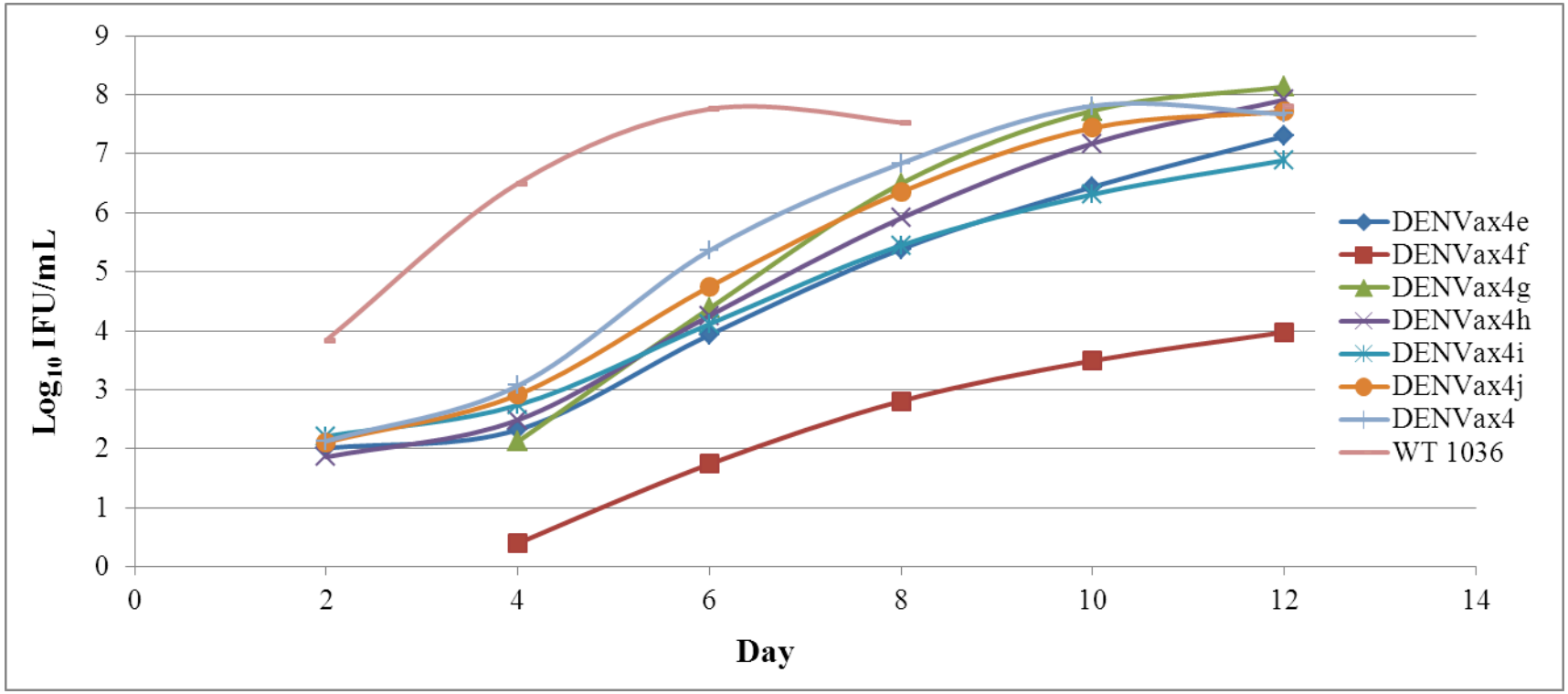

Figure 3.6 **Growth kinetics of DENVax-4 constructs in C6/36 cells.** Constructs were grown in C6/36 mosquito cells for 12 days. Samples taken on designated days were titrated by immunofocus assay. DENVax-4f and DENVax-4g had no IFU formation before day 4. No value is given for WT 1036 on day 10 because of loss of plates during experiment.

Table 3.2 **Viral titers of DENVax-4 constructs tested in mice.** Standard deviations were calculated using titers from duplicate flasks.

	DENVax-4e	
	Vero	**C6/36**
Day	**Log_{10} IFU/mL +/- Standard Deviation**	**Log_{10} IFU/mL +/- Standard Deviation**
2	3.64+/- 0.18	2.01+/- 0.19
4	6.01+/- 0.08	2.32+/- 0.14
6	7.63+/- 0.02	3.93+/- 0.14
8	6.49+/- 0.07	5.38+/- 0.03
10	6.70+/- 0.04	6.44+/- 0.04
12	6.43+/- 0.09	7.30+/- 0.15
	DENVax-4f	
	Vero	**C6/36**
Day	**Log_{10} IFU/mL +/- Standard Deviation**	**Log_{10} IFU/mL +/- Standard Deviation**
2	2.40+/- 0.03	NOT DETECTED
4	4.89+/-0.19	0.40+/-0.35
6	7.26+/-0.05	1.74+/-0.04
8	6.83+/-0.08	2.81+/-0.11
10	6.98+/-0.02	3.49+/-0.15
12	6.58+/-0.03	3.98+/-0.20
	DENVax-4h	
	Vero	**C6/36**
Day	**Log_{10} IFU/mL +/- Standard Deviation**	**Log_{10} IFU/mL +/- Standard Deviation**
2	3.77+/-0.04	1.86+/-0.08
4	6.38+/-0.05	2.49+/-0.02
6	7.46+/-0.05	4.26+/-0.02
8	6.40+/-0.04	5.91+/-0.09
10	6.81+/-0.02	7.18+/-0.18
12	6.11+/-0.02	7.92+/-0.02

value is calculated by counting foci in virus-infected wells containing no sera. Half of this value is the 50% cutoff amount, and the highest dilution of mouse serum containing equal or fewer foci is the value used to calculate the GMT. GMT values for each group in the study were calculated by first taking the GMT of the replicates of each mouse, and then taking the GMT of those values of all the mice in the group. P-values were calculated using a student's t-test with a one-tailed distribution and two variables with unequal variance.

DENVax-4 elicited a 50% plaque reduction rate at a 1:100 serum dilution on day 45 with a GMT of 100 (Figure 3.7). This increased on day 56 to a GMT of 132. DENVax-4f had a 50% plaque reduction rate at approximately 1:100 serum dilution on day 45, which increased to 1:126 on day 56. This suggests that DENVax-4f does not provide a significant increase in immunogenicity when compared to DENVax-4. DENVax-4e had a 50% plaque reduction rate at approximately 1:183 serum dilution on day 45, with a GMT of 183. The GMT increased to 201 on day 56. The p-value for this when compared to DENVax-4 was 0.04 on day 45 and 0.065 on day 56, showing that the difference on day 45 is statistically significant (Table 3.3). Therefore, DENVax-4e had increased immunogenicity in AG129 mice after a single dose to a greater extent than DENVax-4. DENVax-4h had a 50% plaque reduction rate at 1:200 serum dilution. This is a two-fold increase in immunogenicity compared to first generation DENVax-4. On day 56 DENVax-4h had a GMT of 242. The p-values for DENVax-4h on day 45 and day 56 were 0.01 and 0.027 respectively, showing that the data was statistically significant when compared to DENVax-4. The GMT values on day 45 and day 56 for DENVax-4f were close to DENVax-4, and the p-values of 0.33 (day 45) and 0.34 (day 56) showed that the values were not statistically significantly different from DENVax-4.

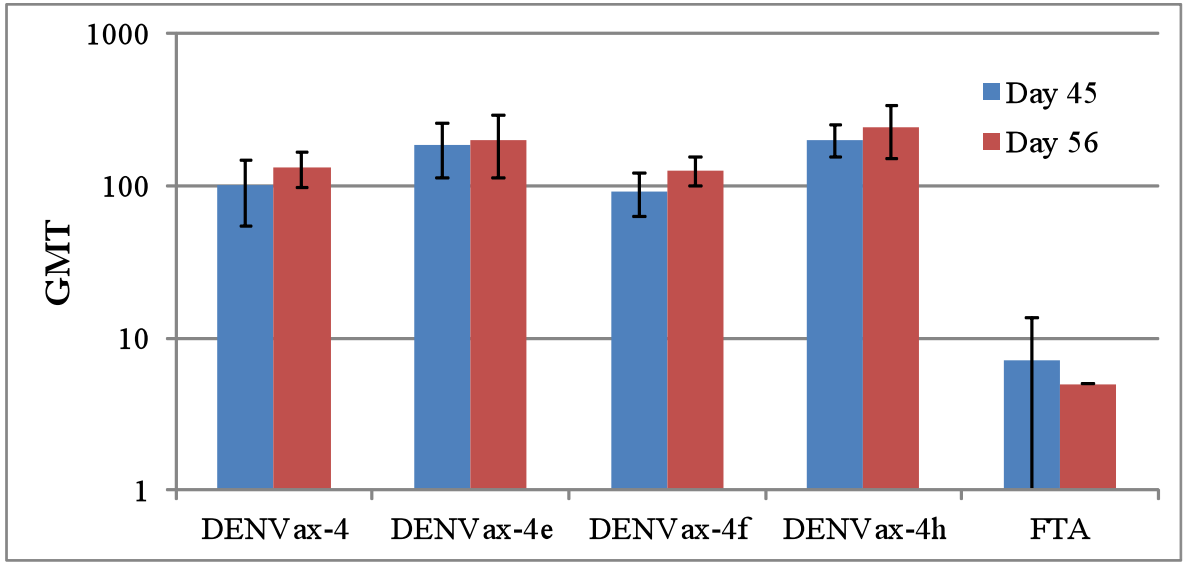

Figure 3.7 **Plot depicting GMT values in AG129 mice at Day 45 and Day 56.** The error bars represent the standard deviation of the data set. GMT values were plotted on a logarithmic scale.

Table 3.3 **Average GMT values and p-values showing significant differences from DENVax-4.** Asterisks mark p-values that are lower than 0.05 and have a confidence interval of 90% or greater.

Vaccine Construct	GMT Day 45	T-Test	GMT Day 56	T-Test
DENVax-4 1st Generation	100.79	0.50 0% Confidence Interval	131.97	0.50 0% Confidence Interval
DENVax-4e	183.79	0.04* 90-95% Confidence Interval	201.58	0.06 80-90% Confidence Interval
DENVax-4f	91.90	0.33 0-50% Confidence Interval	126.99	0.34 0-50% Confidence Interval
DENVax-4h	201.59	0.01* 98% Confidence Interval	242.51	0.03* 95-98% Confidence Interval
FTA (control)	5.00		5.00	

Mouse mortality was also monitored for the duration of the study. FTA control mice died from infection approximately 3-4 days after DENV-2 challenge on day 56 (Figure 3.8). Mice vaccinated with DENVax-4, DENVax-4e, DENVax-4f, and DENVax-4h survived DENV-2 challenge. DENVax-4f and DENVax-4h vaccinated groups each had one mouse succumb to neurovirulence infection approximately 18 days after DENV-2 challenge. Two DENVax-4 vaccinated mice succumbed to neurovirulence approximately 20 days after DENV-2 challenge. All remaining mice were euthanized on day 90.

3.3.7 PLAQUE SIZE

Plaque size is a phenotype used to determine attenuation of a virus. Wild type DENV-4 1036 was used as a control in this experiment, and had an average plaque size of 3.45 mm. All the DENVax-4 second generation constructs had smaller plaque diameters than 1036, indicating an attenuated phenotype (Figure 3.9 and Figure 3.10). DENVax-4e had the smallest plaque size of 0.7 mm, followed by DENVax-4f (1 mm) and DENVax-4h (1.1 mm.). DENVax-4g had the largest plaque size of the constructs (1.85 mm), followed by DENVax-4i (1.8 mm).

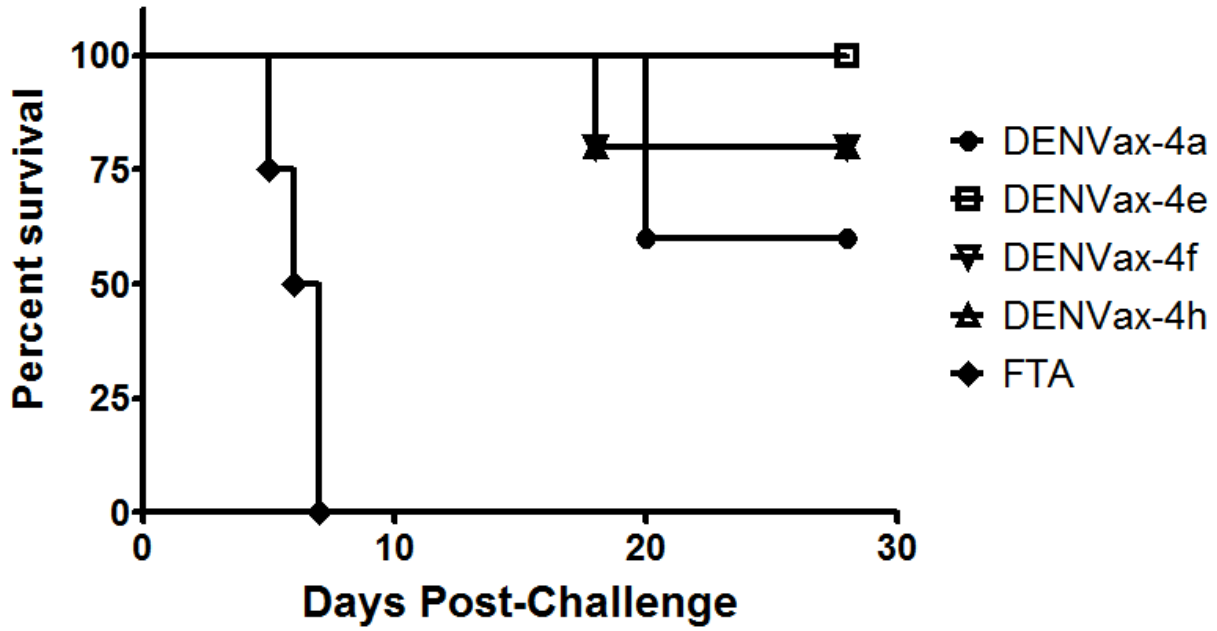

Figure 3.8 **Mortality rate of DENVax-4 vaccinated AG129 mice.** Mice were challenged on day 56 with DENV-2. There were six mice vaccinated with DENVax-4, five vaccinated with DENVax-4e, DENVax-4f, or DENVax-4h, and four vaccinated with FTA (negative control).

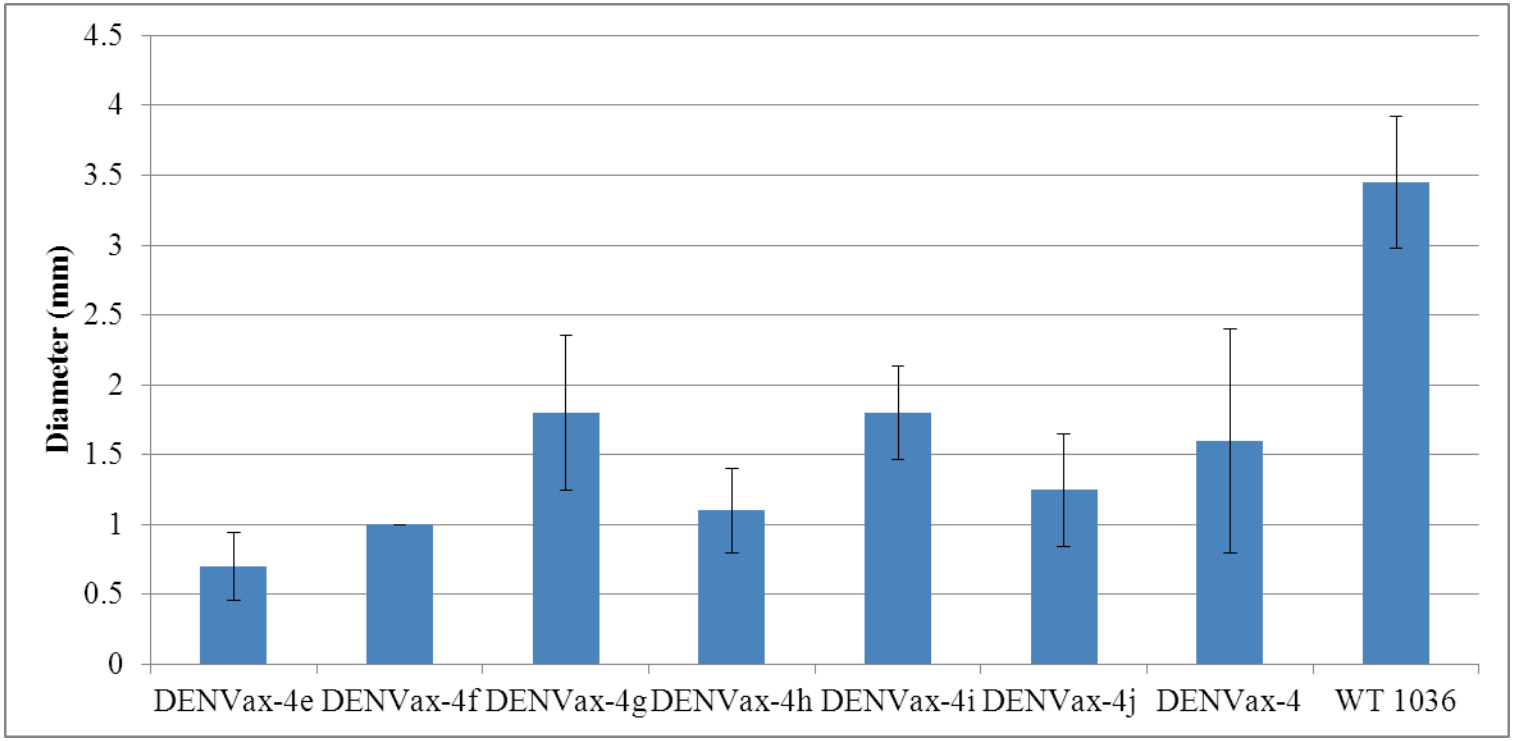

Figure 3.9 **Plaque diameters for DENVax-4 2nd generation constructs.** Values shown are averages of measurements taken from ten different plaques.

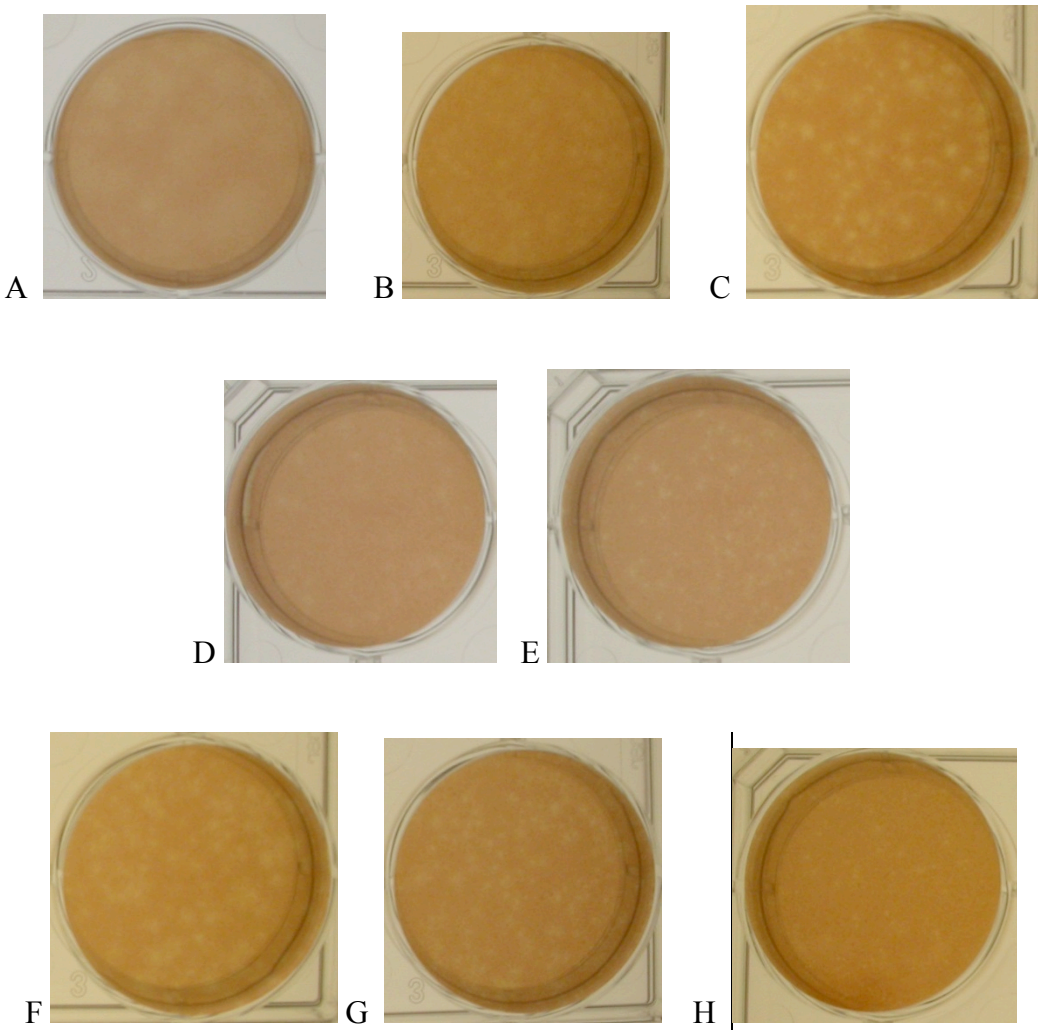

Figure 3.10 **Plaque phenotypes of DENVax-4 2nd generation constructs.** DENV-4 1036(A), DENVax-4 (B), DENVax-4j (C), DENVax-4i (D), DENVax-4h (E), DENVax-4g (F), DENVax-4f (G), and DENVax-4e (H)

3.4 DISCUSSION

Modified live vaccines are used because they often generate a more robust and long-lasting immune response in the host to provide greater protection against pathogen infection. In order to generate this significant immune response the virus must be able to replicate efficiently in the host to stimulate the immune system, but also replicate inadequately so clinical symptoms don't develop. Because of the decreased immunogenicity induced by Inviragen's current DENV-4 vaccine strain, modification is needed to increase growth efficiency in the host in order to generate significant antibody titers. Based on data from previous attempts to reengineer DENVax-4, 6 new potential vaccine constructs were cloned and amplified. All the constructs were tested for improved growth kinetics in Vero cells, decreased replication in C6/36 cells. Phenotypic characterization was done using plaque size and growth kinetics. Genetic characterization was done by full viral RNA genome sequencing.

DENVax-4e, DENVax-4h and DENVax-4i cDNA were successfully cloned, transcribed to infectious RNA, electroporated, and replicated to produce virus in Vero cells. Unlike DENVax-4, passage 2 of these three constructs had to be harvested before the predicted day12 due to complete detachment of the Vero monolayer. DENVax-4e was harvested on day 5 and DENVax-4h and DENVax-4i were harvested on day 7. This indicates that these constructs have a significantly increased growth rate in Vero cells than DENVax-4. However, during the Vero growth kinetics analysis DENVax-4e and DENVax-4h reached similar peak titers to DENVax-4 on day 6. DENVax-4i reached a lower peak titer on day 6 compared to DENVax-4, but reached a similar final titer on day 12. All 3 constructs also reached similar titers to DENVax-4 in C6/36 mosquito cells, showing reduced growth rate compared to DENV-4 1036. DENVax-4e and

DENVax-4h also had significantly decreased plaque size compared to wild type DENV-4 1036, which is an indicator of attenuation.

DENVax-4f, DENVax-4g, and DENVax-4j were generated at by scientists at the CDC. DENVax-4f and DENVax-4g were amplified on Vero cells at Inviragen due to minimal stock. All 3 constructs reached similar titers to DENVax-4 on day 12 in the Vero growth kinetics. However, DENVax-4f had reduced growth rate in Vero cells compared to the other constructs. In C6/36 cells DENVax-4f showed significant attenuation and decreased replication rate compared to the other DENVax-4 constructs, reaching a peak titer of approximately 1e4 PFU/mL after twelve days. The titers of DENVax-4g and DENVax-4j were comparable to DENVax-4.

Because of limited mouse availability only 3 new DENVax-4 constructs were inoculated into mice. DENVax-4e and DENVax-4h were selected because of their similar peak titer values to DENVax-4 in both Vero and C6/36 cells. DENVax-4f was selected because of the significant attenuation shown in C6/36 cells. In AG129 mouse study DENVax-4 was used as a positive control and FTA was used as a negative control. After a single vaccine dose DENVax-4e and DENVax-4h elicited significantly increased neutralizing antibody responses on day 45 compared to DENVax-4. DENVax-4h also elicited significantly increased neutralizing antibody titers on day 56 compared to DENVax-4. This suggests that further testing of DENVax-4h should be completed. Future experiments include preparing a tetravalent formulation of DENVax containing DENVax-4h and testing in AG129 mice, as well as testing monovalent and tetravalent formulations in non-human primates.

CHAPTER 4

SUMMARY

To increase immunogenicity of DENVax-4, we used blind serial passaging of the current DENVax-4 construct and DENVax-4b second generation construct to generate two new DENVax-4 candidates; DENVax-4-P10 and DENVax-4b-P10. DENVax-4b has a different secondary RNA structure than DENVax-4 because of the modifications at the capsid/prM junction (data not shown) (RNAstructure, University of Rochester). Modifying the sequence to be more like DENV-4 instead of DENV-2 would theoretically increase immunogenicity against wild type DENV-4 infection. However DENVax-4b did not show an increase in Vero growth rate even after Vero cell adaptation, indicating further reengineering of DENVax-4 is needed.

Sequencing of the genomes from passages 1 and 10 for DENVax-4 and DENVax-4b revealed two amino acid changes in DENVax-4-P10 (Envelope E417K and NS4A M17L) and one amino acid change in DENVax-4b-P10 (Capsid C107Y). We attempted to optimize DENVax-4 and DENVax-4b by successfully cloning each of these mutations into DENVax-4 or DENVax-4b cDNA to investigate the potential of each of the adaptive mutations for increased growth efficiency in Vero cells. Replication rate in cell culture is an indicator of replication efficiency in the host, which is an important factor in production of neutralizing antibodies. Growth kinetics in Vero cells showed comparable maximum titer to DENVax-4, but higher early growth kinetics was demonstrated in several constructs including DENVax-4e and 4h. As such we tested we

further investigated the immunogenicity of these constructs in AG129 mice. Vaccination in AG129 mice showed an approximately 2-fold increase in immunogenicity in DENVax-4h and an approximately 1.5-fold increase in DENVax-4e.

The envelope protein provides epitopes for neutralizing antibodies, and modifying the sequence to optimize epitope sites for antibody binding to prevent envelope fusion with the endosome would possibly increase immunogenicity. The envelope mutation in DENVax-4h at position 417 is in the conserved portion in the stem region (Figure 4.1). DENV-4 has a different amino acid in this position compared to other flaviviruses. The stem region is in domain III of the E protein where the strongest neutralizing epitope sites exist. Antibodies that bind and neutralize this site prevent the stem region from fusing with the endosome membrane after endocytosis. This is especially important when virus/antibody complexes are taken up by Fcγ receptor-bearing cells. Non-neutralized virions endocytosed by Fcγ receptor-bearing cells is the hypothesis behind antibody dependent enhancement, so strong neutralizing antibodies at this site are critical. However, further investigation needs to be done to test this relationship.

The DENVax-4e Capsid C107Y mutation is located in a cleavage site just before a transmembrane segment that spans the endoplasmic reticulum membrane in the host cell. During viral replication the capsid protein is cleaved from this transmembrane segment by the viral protease (30). The rate of this cleavage affects the rate of cleavage of the prM protein from this segment, and thus affects the rate of virus assembly. Cysteine contains an SH group that can form disulfide bonds. Replacing the cysteine at this cleavage site with a tyrosine may possibly

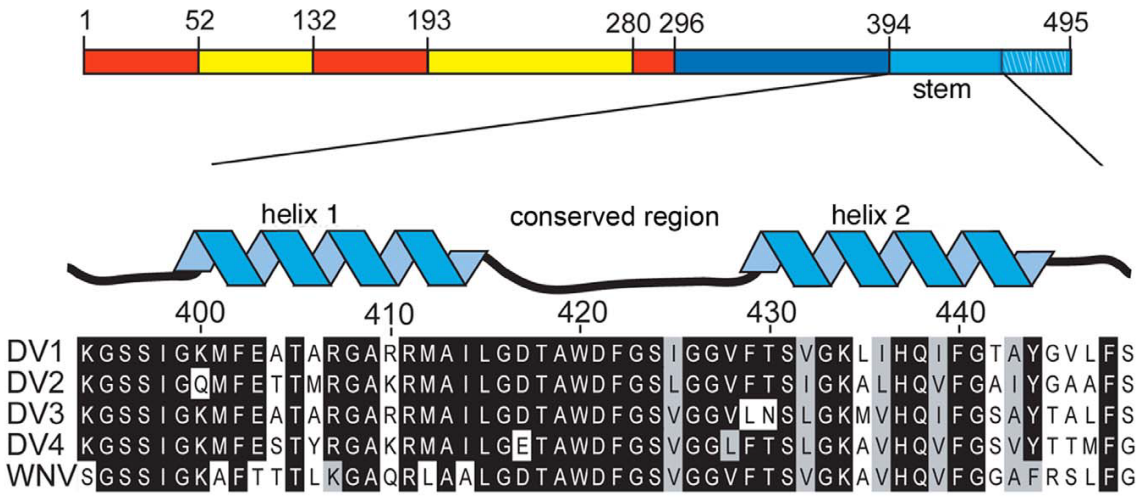

Figure 4.1 **Map of Dengue E protein domains showing a conserved region of each dengue serotype and west nile virus (WNV).** Domain I is red, Domain II is yellow, and Domain III is blue. The fusion loop is at position 98-107.

Schmidt AG et al. (2010) Peptide Inhibitors of Dengue-Virus Entry Target a Late-Stage Fusion Intermediate. *PLoS Pathogens* 6(4): e1000851

prevent disulfide bonds from forming, resulting in more efficient cleavage of the capsid and increased rate of virus assembly.

Improvements in Vero growth kinetics and possible improvements in immunogenicity of DENVax-4e and DENVax-4h warrants further investigation in mouse models utilizing a challenge with more than one DENV-4 isolates. Positive results would then warrant further testing in non-human primates. Tetravalent DENVax formulations containing either DENVax-4e or DENVax-4h should be tested in mice and non-human primates to compare immunogenicity and possible cross reactivity with the other DENVax strains. Full viral genome sequencing of DENVax-4e and DENVax-4h should be completed, and constructs should be genotypically isolated by plaque purification to select for clonal populations.

While current DENVax-4 provides sufficient immunogenicity to protect from infection, it is necessary to continue to adapt this vaccine strain to improve neutralizing antibody titers. Future studies also need to test the ability of DENVax-4 to elicit antibodies that neutralize multiple currently circulating strains of DENV-4. Weak or non-neutralizing antibodies can result in ADE. A dengue vaccine needs to generate long lasting neutralizing antibodies that can protect against multiple strains of dengue virus serotypes, providing lifelong immunity against infection.

REFERENCES

1. Chambers TJ et al. (1990) Flavivirus genome organization, expression, and replication. *Annual Review of Microbiology* 44: 649-688.

2. Welsch S et al. (2009) Composition and Three-Dimensional Architecture of the Dengue Virus Replication and Assembly Sites. *Cell Host and Microbe* 5:365-375.

3. Gutsche I et al. (2011) Secreted dengue virus nonstructural protein NS1 is an atypical barrel-shaped high-density lipoprotein. *PNAS* 108(19): 8003-8008

4. Limjindaporn T et al. (2009) Interaction of dengue virus envelope protein with endoplasmic reticulum-resident chaperones facilitates dengue virus production. *Biochemical and Biophysical Research Communications* 379: 196-200.

5. Butrapet S et al. (2011) Amino acid changes within the E protein hinge region that affect dengue virus type 2 infectivity and fusion. *Journal of Virology* 413: 118-127.

6. Guzman MG et al. (2010) Dengue: a continuing global threat. *Nature Reviews Microbiology*: S7-S16.

7. Lin C-F et al. (2006) Autoimmune Pathogenesis in Dengue Virus Infection. *Viral Immunology* 19(2): 127-132.

8. Fink J et al. (2006) Role of T cells, cytokines, and antibody in dengue fever and dengue hemorrhagic fever. *Review of Medical Virology* 16: 263-275.

9. Hsieh S-C et al. (2011) The C-terminal helical domain of dengue virus precursor membrane protein is involved in virus assembly and entry. *Virology* 410: 170-180.

10. Murphy BR et al. (2011) Immune Response to Dengue Virus and Prospects for a Vaccine. *Annual Review of Microbiology* 29: 587-619.

11. Johansson MA et al. (2011) Models of the impact of dengue vaccines: A review of current research and potential approaches. *Vaccine* 29: 5860-5868.

12. Guy B et al. (2011) From research to phase III: Preclinical, industrial, and clinical development of the Sanofi Pasteur tetravalent dengue vaccine. *Vaccine* 29: 7229-7241.

13. Anderson KB et al. (2011) Interference and Facilitation Between Dengue Serotypes in a Tetravalent Live Dengue Virus Vaccine Candidate. *Journal of Infectious Diseases* 204: 442-450.

14. Thomas SJ et al. (2011) Critical issues in dengue vaccine development. *Current Opinion in Infectious Diseases* 24: 1-9

15. Murrell S et al. (2011) Review of dengue virus and the development of a vaccine. *Biotechnology Advances* 29: 239-247.

16. Osorio JE et al. (2011) Development of DENVax: A chimeric dengue-2 PDK-53-based tetravalent vaccine for protection against dengue fever. *Vaccine* 29: 7251-7260.

17. Butrapet et al. (2006) Determining genetic stabilities of chimeric dengue vaccine candidates based on dengue 2 PDK-53 virus by sequencing and quantitative TaqMAMA. *Journal of Virological Methods* 131: 1-9

18. Brault AC et al. (2011) Replication of the Primary Dog Kidney-53 Dengue 2 Virus Vaccine Candidate in *Aedes aegypti* is Modulated by a Mutation in the 5' Untranslated Region and Amino Acid Substitutions in Nonstructural Proteins 1 and 3. *Vector-borne and Zoonotic Diseases* 11: 1-7.

19. Huang C Y-H et al. (2000) Chimeric Dengue Type 2 (Vaccine Strain PDK-53)/Dengue Type 1 Virus as a Potential Candidate Dengue Type 1 Virus Vaccine. *Journal of Virology* 74: 3020-3028.

20. Butrapet S et al. (2000) Attenuation Markers of a Candidate Dengue Type 2 Vaccine Virus, Strain 16681 (PDK-53) are Defined by Mutations in the 5' Noncoding Region and Nonstructural Proteins 1 and 3. *Journal of Virology* 74: 3011-3019.

21. Huang C Y-H et al. (2003) Dengue 2 PDK-53 Virus as a Chimeric Carrier for Tetravalent Dengue Vaccine Development. *Journal of Virology* 77: 11436-11447.

22. Kinney RM et al. (1997) Construction of Infectious cDNA Clones for Dengue 2 Virus: Strain 16681 and Its Attenuated Vaccine Derivative, Strain PDK-53. *Journal of Virology* 230: 300-308.

23. Costa RL et al. (2012) Comparative evolutionary epidemiology of dengue virus serotypes. *Infection, Genetics, and Evolution* 12: 309-314.

24. Pereira de Souza R et al. (2011) Dengue Virus Type 4 Phylogenetics in Brazil 2011: Looking beyond the veil. *PLoS Neglected Tropical Diseases* 5: e1439

25. Lanciotti RS et al. (1997) Molecular evolution and phylogeny of dengue-4 viruses. *General Virology* 78: 2279-2286.

26. Fried JR et al. Serotype-Specific Differences in the Risk of Dengue Hemorrhagic Fever: An Analysis of Data Collected in Bangkok, Thailand from 1994 to 2006. *PLoS Neglected Tropical Diseases* 4: e617.

27. Mota J et al. (2002) Phylogenetic analysis of the envelope protein (domain III) of dengue 4 viruses. *Salud Publica de Mexico* 44: 228-236.

28. Foster JE et al. (2003) Molecular evolution and phylogeny of dengue type 4 virus in the Caribbean. *Journal of Virology* 306: 126-134.

29. Rossi SL et al. (2012) Genetic and phenotypic characterization of sylvatic dengue virus type 4 strains. *Journal of Virology* 423: 58-67.

30. Knipe, D. M. & Howley, P. M. (2007). *Field's Virology* (5th ed.). Philadelphia, PN: Lippincott-Raven Publishers.

31. Sanchez-Vargas I et al. (2009). Dengue Virus Type 2 Infections of *Aedes aegypti* Are Modulated by the Mosquito's RNA Interference Pathway. *PLoS Pathogens* 5(2): e1000299.

32. Diaz-Nieto LM et al. (2013) Geographical Limits of the South Eastern Distribution of *Aedes aegypti* (Diptera, Culicidae) in Argentina. *PLoS Neglected Tropical Diseases* 7(1): e1963.

33. Flipse J et al. (2012) Molecular Mechanisms Involved in Antibody-Dependent Enhancement of Dengue Virus Infection in Humans. *Traffic*: 1-11

34. Hughes HR et al. (2012) Manipulation of immunodominant dengue virus E protein epitopes reduces potential antibody-dependent enhancement. *Virology Journal* 9:115

35. Chan KR et al. (2011) Ligation of Fc gamma receptor IIB inhibits antibody-dependent enhancement of dengue virus infection. *PNAS* 108(30): 12479-12484

36. Jessie K et al. (2004) Localization of Dengue Virus in Naturally Infected Human Tissues, by Immunohistochemistry and In Situ Hybridization. *Journal of Infectious Diseases* 189: 1411-1418

37. Rajapakse S et al. (2012) Treatment of dengue fever. *Infection and Drug Resistance* 5: 103-112

38. Wahala MP et al. (2011) The Human Antibody Response to Dengue Virus Infection. *Viruses* 3: 2374-2395

39. Schmidt AG et al. (2010) Peptide Inhibitors of Dengue-Virus Entry Target a Late-Stage Fusion Intermediate. *PLoS Pathogens* 6(4): e1000851

40. Huang CY-H et al. (2009) The dengue virus type 2 envelope protein fusion peptide is essential for membrane fusion. *Journal of Virology* 396: 305-315

41. Byrd CM et al. (2013) A Novel Inhibitor of Dengue Virus Replication That Targets the Capsid Protein. *Antimicrobial Agents and Chemotherapy* 57(1): 15-25

42. Watanabe S et al. (2012) The Magnitude of Dengue Virus NS1 Protein Secretion Is Strain Dependent and Does Not Correlate with Severe Pathologies in the Mouse Infection Model. *Journal of Virology* 86(10): 5508-5514

43. Le Breton M et al. (2011) Flavivirus NS3 and NS5 proteins interaction network: a high-throughput yeast two-hybrid screen. *BMC Microbiology* 11: 234

44. Cleaves GR et al. (1981) Identification and Characterization of Type 2 Dengue Virus Replicative Intermediate and Replicative Form RNAs. *Journal of Virology* 111: 73-83

45. Tsai W-Y et al. (2012) C-Terminal Helical Domains of Dengue Virus Type 4 E Protein Affect the Expression/Stability of prM Protein and Conformation of prM and E Proteins. *PLoS One* 7(12): e52600

46. Korrapati AB et al. (2012) Adenovirus Delivered Short Hairpin RNA Targeting a Conserved Site in the 5' Non-Translated Region Inhibits All Four Serotypes of Dengue Viruses. *PLoS Neglected Tropical Diseases* 6(7): e1735

47. Smith KM et al. (2012) Testing of Novel Dengue Virus 2 Vaccines in African Green Monkeys: Safety, Immunogenicity, and Efficacy. *American Journal of Tropical Medicine and Hygiene* 87(4): 743-753

48. Sabchareon A et al. (2012) Protective efficacy of the recombinant, live-attenuated, CYD tetravalent dengue vaccine in Thai schoolchildren: a randomized, controlled phase 2b trial. *Lancet* 380: 1559-1567

49. D'Arcy A et al. (2006) Purification and crystallization of dengue and West Nile virus NS2B-NS3 complexes. *Structural Biology and Crystallization Communications* 62:157-162

50. Falgout B et al. (1989) Proper Processing of Dengue Virus Nonstructural Glycoprotein NS1 requires the N-Terminal Hydrophobic Signal Sequence and the Downstream Nonstructural Protein NS2a. *Journal of Virology* 63(5): 1852-1860

51. Lundin M et al. (2003) Topology of Membrane Associated Hepatitis C Virus Protein NS4B. *Journal of Virology* 77(9): 5428-5438

52. Mackenzie JM et al. (1998) Subcellular Localization and Some Biochemical Properties of the Flavivirus Kunjin Nonstructural Proteins NS2A and NS4A *Journal of Virology* 245: 203-215

53. Hidari K et al. (2011) Dengue virus receptor. *Tropical Medicine and Health* 39(4): 37-43

54. Bhamarapravati N et al. (1987) Immunization with a live attenuated dengue-2-virus candidate vaccine (16681-PDK 53): clinical, immunological and biological responses in adult volunteers. *Bulletin of the World Health Organization* 65(2): 189-195

55. Mulhern K (2010) DENVax Live Attenuated Chimeric Dengue Vaccine. *Master's Thesis, Colorado State University*

56. Sun W et al. (2003) Vaccination of Human Volunteers with Monovalent and Tetravalent Live-Attenuated Dengue Vaccine Candidates. *American Journal of Tropical Medicine and Hygiene* 69(Suppl 6): 24-31

57. Blaney JE et al. (2010) Targeted Mutagenesis as a Rational Approach to Dengue Virus Vaccine Development. *Current Topics in Microbiology and Immunology 338*: 145-158

58. Johnson AJ et al. (1998) New Mouse Model for Dengue Virus Vaccine Testing. *Journal of Virology* 73(1): 783-786