THESIS

DENVAX LIVE ATTENUATED CHIMERIC DENGUE VACCINE

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY KAITLYN MULHERN ENTITLED DENVAX LIVE ATTENUATED CHIMERIC DENGUE VACCINE BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

DENVAX LIVE ATTENUATED CHIMERIC DENGUE VACCINE

Dengue viruses are endemic in regions inhabited by roughly one half of the world's population and can cause symptoms ranging from a headache and fever to even death. There is no vaccine to prevent infection by dengue viruses and the only current protection and prevention is mosquito control. The dengue viruses and the *Aedes aegypti* mosquito that carries them are spreading to more areas of the world and are therefore posing an even greater threat to public health. There is an urgent need for an effective vaccine that confers protection against infection by all four dengue serotypes. Several companies are currently developing dengue vaccines using different technologies including a live attenuated virus vaccine, DNA vaccine, and a chimeric vaccine in the yellow fever virus vaccine backbone. Inviragen, Inc., a biotech company, in collaboration with the Center for Disease Control and Prevention (CDC), is developing a chimeric live attenuated virus vaccine that is currently in clinical testing.

The Inviragen/CDC dengue vaccine is based on an attenuated dengue-2 virus, called PDK-53, which was developed by passaging the virus 53 times in primary dog kidney cells (PDK). The attenuating mutations in the dengue-2 PDK-53, which are in the nonstructural genes of the virus, are well defined and characterized. This is essential in a

live attenuated vaccine in order to monitor the stability of the mutations throughout vaccine manufacture and development. The four separate serotype-specific vaccine viruses are constructed by inserting the appropriate structural E and prM gene sequences into the nonstructural genes of the attenuated dengue-2 PDK-53 backbone. The phenotypic properties including plaque size, temperature sensitivity, peak titers, efficiency of replication, and neurovirulence, showed that the chimeric viruses for each serotype were attenuated in comparison to the wild type parental viruses. Tetravalent formulations with the chimeric D2/1, D2/3 and D2/4 viruses as well as the D2-PDK53 virus were tested for antibody responses in AG129 mice.

Two separate projects involving Inviragen's dengue vaccine, DENVax, are discussed in this thesis. In the first project, assays were developed to purify and quantify the residual host cell DNA in the vaccine using real time qPCR. Initially, the purification of host cell DNA present in research grade vaccine lots was optimized and internal controls were generated to monitor the efficiency of DNA extraction from the samples. The purified DNA was then amplified by PCR and quantified using two fluorescence-based methodologies, SYBR Green and TaqMan. The SYBR Green method was used to test the virus samples and reproducible results were obtained. The TaqMan assay allows for multiplexing, which allows simultaneous detection of two PCR products; Vero cell DNA present in virus samples and an internal control. The multiplexing TaqMan reactions need to be further optimized. In the second project, three separate "second generation" DENVax4 viruses were designed and generated. Of the three, two viable infectious viruses were generated and tested for phenotypic growth *in vitro*, and sequenced. These

viruses grew to similar titers and phenotypes to the existing DENVax4, and immunogenicity testing in AG129 mice is planned.

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CHAPTER 1

BACKGROUND ON DENGUE VIRUSES AND VACCINE DEVELOPMENT

1.1. INTRODUCTION

Dengue viruses are members of the genus *Flavivirus* and family *Flaviviridae*. Other members of the *Flavivirus* genus include Japanese encephalitis, yellow fever, and tick-borne encephalitis viruses (*1*). *Flaviviruses* are 45-50 nm in diameter enveloped RNA viruses that contain a single-stranded positive sense genome (Figure 1.1). The genome is approximately 11kb in length and has a capped 5' end with no 3' poly (A) tail. The genome arrangement is 5'UTR-C-prM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'UTR. The structural proteins are the capsid (C), premembrane/membrane (prM/M), and envelope (E) (*2*). The capsid (C) protein is a small highly basic protein, which forms the nucleocapsid along with the genomic RNA (*3*). The capsid has an unstructured N terminus that might play a role in RNA binding (*4*). Sequence homology between the different flavivirus capsid proteins is low, but they have conserved hydrophobic and hydrophilic sections.

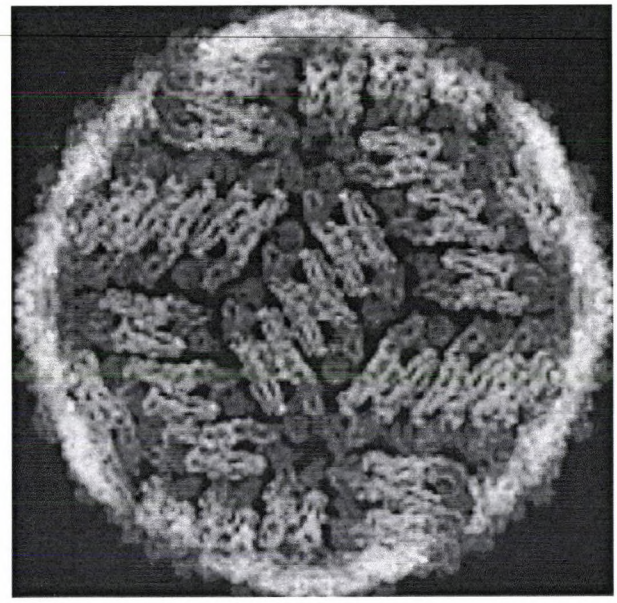

Figure 1.1 **Dengue virus.** Cryoelectron microscopy was used to visualize mature dengue virion. Photo courtesy of Dr. Richard Kuhn at Purdue University.

The envelope (E) protein is the major structural protein of the flavivirus virion. It contains three domains, Domain I, which is a centrally located β barrel, Domain II, which contains a dimerization region and the important fusion peptide, and Domain III, which contains the receptor-binding activity (*5*). In the mature virion, the envelope (E) proteins exist as homodimers. In that conformation the fusion peptide is hidden and inaccessible. When the virus particle attaches to a host cell it is taken in by receptor-mediated endocytosis. The low pH environment of the endosome causes a conformational change to occur in the E protein, which results in irreversible trimer formation. This trimerization event exposes the fusion peptide and allows fusion of the cellular membrane with the viral membrane (*3*). This resulting fusion pore grows and eventually allows the viral genome to be released into the cytoplasm of the host cell. The envelope protein plays a major role in membrane fusion, receptor binding and virion assembly. The envelope (E) structural protein is also the target for neutralizing antibodies (*2*).

The prM protein is the glycoprotein precursor of the membrane (M) structural protein. Furin cleaves the prM protein into the M protein in the *trans* Golgi network, late in secretion (*5*). The cleavage of prM releases the pr fragment and causes the E protein to form homodimeric complexes. If the prM protein is not cleaved it prevents fusion of the E protein during virus maturation. The prM protein is thought to protect the E protein from pH-induced reorganization before fusion is supposed to occur upon viral entry (*3*).

The nonstructural proteins are NS1-NS5 and are involved in viral RNA replication (*3*). The NS1 nonstructural protein is a glycoprotein that is released from the surface of cells infected by a flavivirus (*6*). NS1 is important for viral replication but its exact function is

unknown (*3*). The NS2A, NS4A, and NS4B proteins are all hydrophobic and not as well characterized as the other nonstructural proteins. They are hypothesized to help localize the vRNA and viral proteins to the areas of RNA synthesis and virion assembly (*5*). The NS3 protein has a nucleotide triphosphatase and RNA helicase activity located at the C terminus, functions that are important for replication of the viral RNA (*7*). NS3 also has a serine protease at the N terminus of the protein, which requires the NS2B cofactor for complete function (*8*). The NS5 protein is the viral RNA dependent RNA polymerase (*5*). The N terminus of this protein also contains a methyl transferase domain that is involved in formation of the RNA cap structure (*3*).

1.2. Infection with Dengue Virus

There are four separate serotypes of dengue virus, known as DEN-1 through DEN-4. Dengue viruses are mosquito-borne pathogens transmitted from human to mosquito to human primarily by the bite of an infected *Aedes aegypti* mosquito, which is found in most tropical and subtropical regions of the world (Figure 1.2). The *Aedes albopictus* mosquito is also capable of transmitting the virus although less efficiently as *Aedes aegypti* (*9*).

After infection with one of the four serotypes, dengue fever can develop, which is characterized by a high fever, headache, rash, muscle pain and vomiting. Infection by one of the dengue serotypes provides lifelong protection against re-infection with the same serotype but does not cross protect against infection from the other serotypes. A

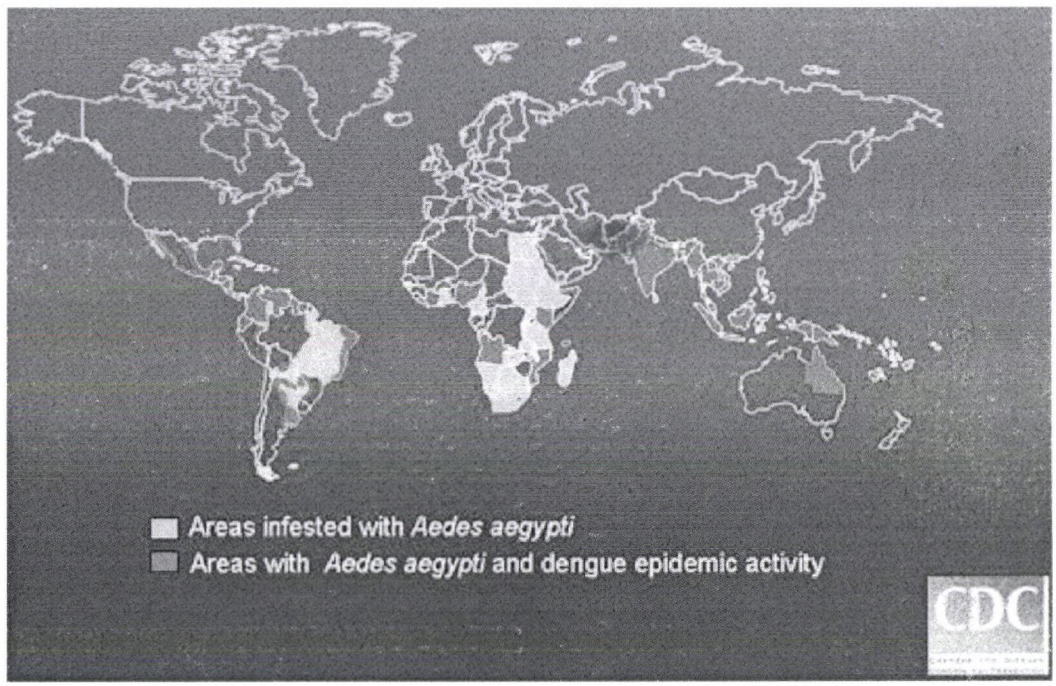

Figure 1.2 **World distribution of Dengue Fever in 2005**.

person will need to be exposed to all four serotypes before complete immunity from developing dengue fever is accomplished (*10*). More severe forms of the disease called Dengue Hemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS) can occur by an immunopathological response generated by secondary infection of dengue virus. Symptoms of DHF and DSS include high fever, abdominal pain, hemorrhaging and sometimes circulatory failure, caused by vascular leakage, and death (*10*). Dengue hemorrhagic fever or dengue shock syndrome results in death in 20% of the untreated cases (*11*). It is hypothesized that the development of the more severe DHF/DSS is due to antibody dependent enhancement (ADE) of infection. This occurs when cross-reactive antibodies generated by the first infection bind to the new dengue virus serotype from the second infection, but do not neutralize it (*12*). The antibody/virus complexes can bind to Fc bearing monocytes and help the virus enter the cell more quickly, increasing levels of viremia and inducing the expression of high levels of cytokines and other inflammatory mediators. This causes an immunopathological response that damages blood vessels and can lead to shock and major organ damage.

1.3. PREVALENCE

About 3.5 billion people in the world are at risk of dengue infection, which is about half of the world's population. There are estimated to be 50-100 million cases of dengue fever each year and about 500,000- 2 million cases of DHF/DSS each year (www.pdvi.org). Figure 1.2 shows the world distribution of Dengue Fever in 2005. The

yellow represents the countries infested with *Aedes aegypti* and the red represents the countries with the mosquito and where dengue is epidemic. The first reported cases of dengue fever occurred in Africa, Asia and the Americas between the years of 1779-1780 (*10*). Since then the virus has spread and is endemic throughout the tropical and subtropical regions of the world, causing epidemic outbreaks. The habitat of *Aedes aegypti* is expanding, which is contributing to the spread of dengue. This spread is thought to be due to modern transportation, urbanization in countries with *Aedes aegypti* mosquito, and the absence of effective mosquito control efforts (*13*). Currently, mosquito control is the only way to prevent dengue infection but it has not been sustained. There is an enormous need for a vaccine that will prevent dengue infection and elicit a long lasting immune response for protection against all four serotypes.

The epidemiology of dengue is different in various parts of the world. In many regions of the world the dengue vaccine would be given primarily to adults. On the other hand, the vaccine would be given primarily as a childhood immunization in Southeast Asia where most adults have already been infected by all four serotypes and are protected. During pregnancy, the antibodies of the mother are passed to the developing fetus. When the child is born and throughout the first few months of its life, the infant will benefit from maternal protection, a mechanism that provides protection specifically against the dengue serotypes that the mother had previously been infected with. The neutralizing antibodies protect the child until about six months of age. At this point the child needs to be vaccinated as the maternal antibodies present in their body are at low levels, thus increasing their risk of developing DHF or DSS (*9*).

1.4. Vaccine Candidates

Dengue vaccines currently being developed include live attenuated virus vaccine (LAV), mutated, and yellow fever backbone chimeric vaccines. The Walter Reed Army Institute of Research (WRAIR) developed a combination of live attenuated viruses by serial passage, each using separate wild type strains, in tissue culture cells. Preclinical and Phase 1 studies were completed, showing positive results (*14*). This vaccine formulation by WRAIR is now in Phase 2 clinical testing through GlaxoSmithKline. The mutations in the virus vaccine candidates, however, have not been identified and characterized, which makes genetic analysis and control difficult during testing and production. Mahidol University in collaboration with Aventis Pasteur started research and development of a tetravalent LAV dengue vaccine. The tetravalent vaccine was tested in multiple phase 1 and phase 2 studies (*15-18*). Further development of these vaccines was stopped due to generation of dengue-like symptoms. The dengue-3 vaccine virus was identified in the serum of sick patients and therefore attributed to the cause of the symptoms (*16*). A tetravalent recombinant LAV vaccine in the yellow fever vaccine backbone was developed by Acambis/Sanofi Pasteur and is currently in Phase 2b efficacy testing (*19*). The vaccine is based on the backbone for the yellow fever vaccine (YF 17D) replication genes and contains the envelope genes of the four dengue serotypes. The National Institute of Allergy and Infectious Diseases (NIAID) is developing a recombinant LAV tetravalent vaccine by deleting 30 nucleotides in the 3' UTR of the dengue virus (*9*). The vaccine by NIAID is currently in preliminary Phase I testing. A

DNA based vaccine is being developed by the Navy Medical Research Center in which the complementary DNA of the virus RNA genome will be injected into animal models for clinical testing. Hawaii Biotech is developing a recombinant subunit vaccine using genetically engineered proteins from each of the dengue virus serotypes (*9*).

The vaccine developed by Inviragen/Centers for Disease Control and Prevention (CDC), is a recombinant live attenuated virus vaccine based on the DEN-2 PDK-53 backbone. The backbone used for each chimeric virus was attenuated by serial passed in primary dog kidney (PDK) cells. It is designed to elicit sterilizing, long lasting immunity in the recipient, which is essential to vaccine success. The vaccine must generate limited viremia and be attenuated in humans so as to eliminate the development of dengue symptoms after immunization. The transmissibility by mosquitoes must also be reduced to eliminate the possibility of transmitting the vaccine viruses in nature. Additionally, the vaccine must be capable of delivery at low cost, since the majority of people living in endemic areas are in underdeveloped regions of the world with limited resources and access to healthcare. Ideally, the attenuating mutations will be well defined to control the genetic integrity throughout development and monitor any other mutations that occur. Lastly, it must elicit a balanced immune response to all four dengue serotypes to eliminate the risk of developing the more severe form DHF/DSS due to sub-optimal protection to one serotype (*9*).

1.5. INVIRAGEN/CDC DENGUE VACCINE

The Mahidol University in Bangkok, Thailand isolated four wild type dengue virus serotypes, DEN-1 16007, DEN-2 16681, DEN-3 16562, and DEN-4 1036 from infected patients. The DEN-1 16007 and DEN-2 16681 parental viruses were isolated from two patients that had DHF/DSS in Thailand (*12*) and were passaged *in vitro* through primary dog kidney (PDK) cells to generate attenuated vaccine virus candidates. The DEN-3 serotype was isolated from a patient with DHF/DSS in the Philippines and the DEN-4 was isolated from a child with DHF/DSS in Indonesia (*12*). The nomenclature used after serial passage in PDK cells is PDK-13 (13 passages in PDK cells) for DEN-1 and PDK-53 (53 passages in PDK cells) for DEN-2. The four attenuated viruses were tested in nonhuman primates and went as far as human clinical trials in the US and Thailand (*20*). The DEN-2 PDK-53 vaccine virus was shown to elicit neutralizing antibodies lasting up to 2 years in adult volunteers (Figure 1.3).

1.6. MAKING CDNA CLONES

The Centers for Disease Control and Prevention (CDC) in Fort Collins, CO collaborated with Mahidol University to genetically characterize DEN-2 PDK-53. Richard Kinney and Claire Huang at the CDC constructed infectious cDNA clones for the wild type DEN-2 16681, and its vaccine derivative PDK-53 (*21*). DEN-2 genomic RNA was isolated from a Mahidol University virus stock and made into cDNA by amplification using reverse transcriptase/polymerase chain reaction (RT/PCR). The cDNA was cloned

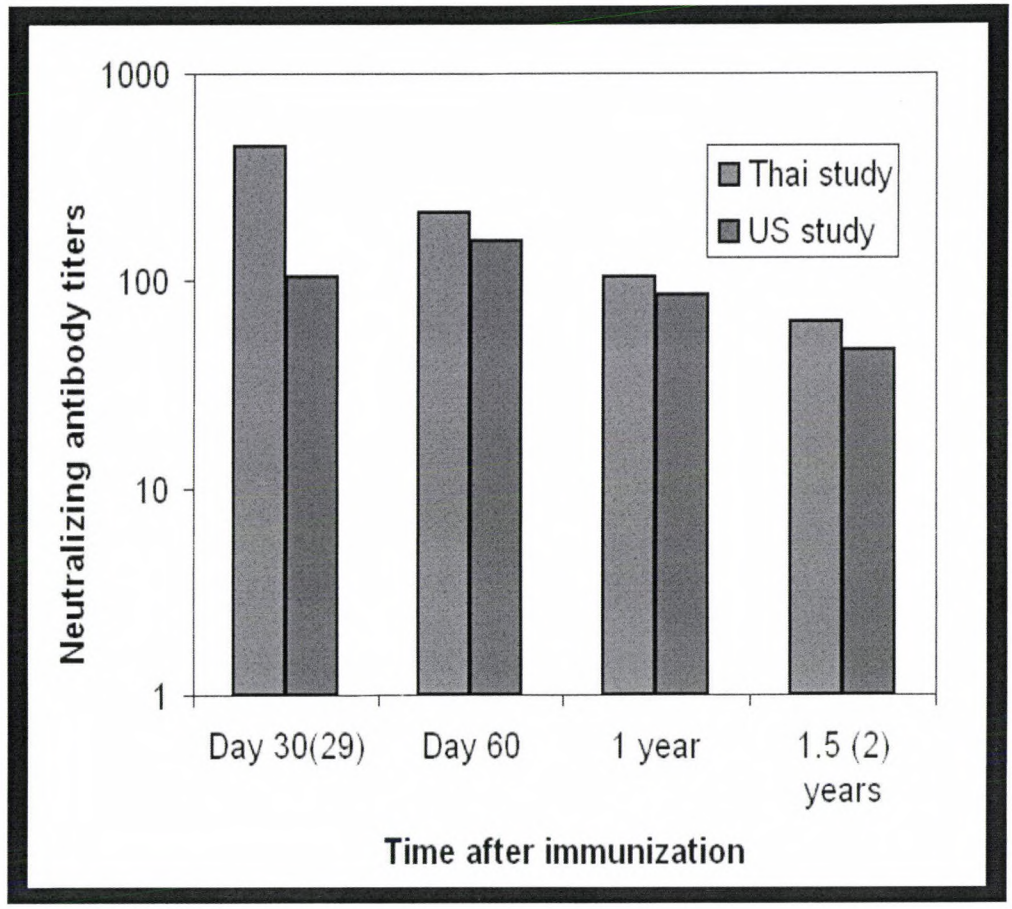

Figure 1.3 **Phase I clinical trial of PDK-53 virus in US and Thailand.** Neutralizing antibody titers after immunization with Dengue 2 PDK-53 vaccine virus tested by plaque reduction assay. Vaughn, D. W (1996) Testing of a dengue 2 live-attenuated vaccine (strain 16681 PDK 53) in ten American volunteers, *Vaccine 14*, 329-336.

into a TA vector and then electroporated into competent *E. coli* cells for growth on agar plates. The plasmid DNA was then purified and sequenced. Sequencing results showed nine nucleotide mutation differences in the PDK-53 virus when compared to its parental 16681 strain (Figure 1.4) (*21*). Three of the observed mutations were silent and did not change the amino acid sequence of the PDK-53 virus (shown with the ^ in the figure). The other six mutations in the PDK-53 virus produced a change in the amino acid sequence. They found a mixed population of viruses; some of the PDK-53 viruses had the parental glutamic acid (E) at the NS3 amino acid position 250, and others carried a mutation to valine (V). These two virus strains were called PDK53-E and PDK53-V.

1.7. PHENOTYPIC ATTENUATION MARKERS

The DEN-2 parental and mutated PDK-53 viruses were evaluated *in vitro* and *in vivo* to characterize the phenotypic markers associated with attenuation. The parameters studied in these experiments were plaque size, growth rate, temperature sensitivity and neurovirulence (*22*). A plaque titration was done in LLC-MK_2 (rhesus monkey kidney cells) to examine the size of the plaques generated by each virus. The PDK-53 virus had smaller plaques than the parental 16681, which could indicate attenuation when compared to the wild type strain. Replication rates were determined by examining peak viral titers in LLC-MK_2 and C6/36 *Aedes albopictus* mosquito cells. The PDK-53 virus replicated less efficiently in both cells types than the parental 16681 DEN-2 strain, another sign of attenuation *in vitro* (data not shown). Temperature sensitivity was tested

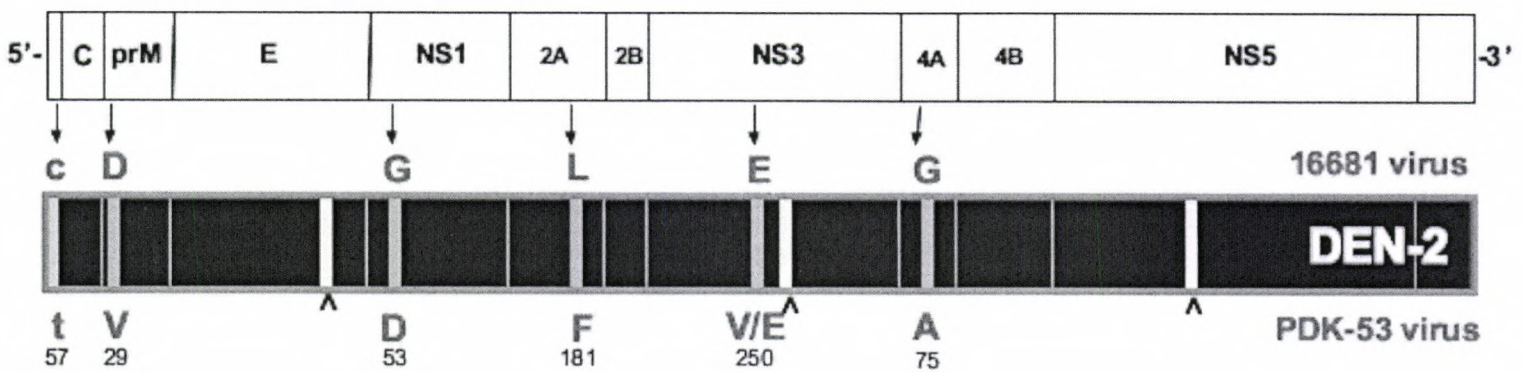

Figure 1.4 **Amino acid changes in PDK-53 virus compared to the parental 16681 virus.** Nine nucleotide changes were observed between the dengue 2 PDK-53 and the parental 16681 virus. The silent mutations are shown by (^).

in LLC-MK_2 cells and titers were compared when grown at 37°C versus 39°C. The PDK-53 virus titers were reduced by 97%-99.98% when grown at 39°C compared to 37°C (depending on whether the PDK-53 strain carried the E or V NS3-250 mutation), whereas the parental 16681 titers were reduced by 89%-90% by growth at 39°C compared to 37°C. This showed that the PDK-53 virus was more temperature sensitive than the DEN-2 16681 parental virus, and therefore more attenuated. Newborn ICR mice were injected intracranially (IC) with 10^4 PFU of either the PDK-53 DEN-2 virus or the parental wild type DEN-2 16681. The 16681 virus caused 80-90% mortality in suckling mice whereas the PDK-53 vaccine virus IC-challenged mice all survived without significant weight loss (*21*). In summary, after investigating the phenotypic characteristics associated with attenuation, this study demonstrated that the DEN-2 PDK-53 virus was more attenuated than the parental 16681 virus.

1.8. Attenuating Mutations

To identify the genetic determinants of attenuation, Butrapet et al. analyzed panels of recombinant DEN-2 16681 and PDK-53 viruses (*22*). Eighteen different chimeric viruses were constructed by incorporating portions of the attenuated PDK-53 virus genome into the DEN-1 16681 parental virus as a genetic backbone and vice versa. The parameters studied were plaque size, replication in C6/36 cells, and neurovirulence in newborn mice, temperature sensitivity, and *in vitro* growth rate. The mutations in the 5' noncoding region nucleotide 57-T (5'NC-57-T) and in NS1 amino acid 53-Asp (NS1-53-Asp) were

shown to slow the replication of the virus in C6/36 cells and decrease its virulence in mice. The mutations at NS1-53-Asp and NS3 amino acid 250-Val (NS3-250-Val) were necessary for the temperature sensitivity of the PDK-53 virus. The small plaque size of the PDK-53 vaccine virus was a result of attenuating mutations in the 5'-NC-57-T, NS1-53-Asp, and NS3-250-Val loci (*22*). The genetic markers of attenuation are shown in Figure 1.5, with the red arrows pointing to the attenuating mutation sites in the 5'NC region, NS1, and NS3. There were no mutations in the structural genes C, M or E of the PDK-53 vaccine virus. Therefore, the PDK-53 virus appeared to be ideal to serve as the backbone for the generation of the chimeric viruses (*21*).

1.9. Chimeric vaccine cloning and Characterization

The envelope protein is the immunogenic determinant in dengue infection. Since the structural genes remained intact in DEN-2 PDK-53, it was hypothesized that long lasting immunity would still be generated if you substitute the structural genes from DEN-1, 3, and 4 serotypes into the DEN-2 PDK53 backbone. To test this hypothesis, panels of DEN-2/1 chimeric viruses were constructed (*23*). These DEN-2/1 chimeric viruses contained the nonstructural genes of DEN-2 16681 parental virus or its vaccine derivative PDK-53, with the structural genes of DEN-1 16007 parental virus or its vaccine derivative PDK-13. The phenotypic characteristics for attenuation were analyzed for each chimeric virus, including plaque size, temperature sensitivity, peak titers, and neurovirulence in newborn mice. The chimeric viruses containing the structural genes of

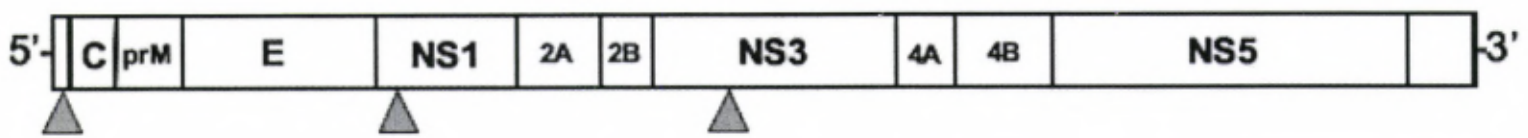

Figure 1.5 **Genetic markers of attenuation in PDK-53 backbone.** The three mutations determined to be sufficient for attenuation are shown by the red arrows.

wild type DEN-1 16007 and nonstructural genes of DEN-2 PDK-53 retained the phenotypes of attenuation (*23*). Since the chimeric DEN-2/1 virus retained the phenotypic characteristics of attenuation similar to the DEN-2 PDK-53 virus, Huang et al constructed chimeric viruses using the DEN-2 PDK-53 backbone for the other dengue serotypes.

The cDNA clones of PDK-53 produced two separate variants that were mentioned previously (Figure 1.4). The PDK53-E contained eight out of the nine mutations with the conserved NS3-250-Glu of parental 16681. This variant had less temperature sensitivity and replicated better in C6/36 cells than the uncloned PDK-53. The PDK53-V variant contained all nine of the mutations including the NS3-250-Val. It showed smaller plaques, decreased replication, and increased temperature sensitivity. The NS3-250-Glu→Val mutation affects the virus replication, plaque phenotype, temperature sensitivity, and replication rate *in vivo* (*21*). Both variants were used for the following studies to determine the NS3-250-Glu/Val effect on attenuation.

Chimeric viruses were constructed by inserting the structural genes of wild type DEN-3 16562 and DEN-4 1036 into the genetic backbones of either wild type DEN-2 16681, PDK53-E or PDK53-V. The chimeric D2/3 viruses had many mutations in the working seeds that were not in the cDNA infectious clone. A single mutation seemed to stabilize the viruses, at nucleotide 1968 which changed the amino acid at E-345 from histidine to leucine. This mutation was introduced to all the cDNA infectious clones and resulted in stable constructs (*24*).

The D2/4 chimeric viruses with the attenuated PDK53-E or -V backbone were not viable when grown in C6/36 cells, but the D2/4 chimeras with the wild type DEN-2 16681 backbone generated viable virus. The D2/4 chimeras containing the DEN-2 16681 backbone were passaged one more time in C6/36 cells and then passaged five times in LLC-MK_2 cells. The viruses were sequenced and three mutations were determined to aid in efficient mammalian cell growth and viability of the viruses. The three mutations were at C-100 (Arg to Ser), E-364 (Ala to Ala/Val mixed), and E-447 (Ile to Val). These mutations were incorporated into the cDNA of the chimeric D2/4 constructs in the attenuated backbone and then transfected into LLC-MK_2 cells. Viable D2/4 chimeric viruses with the attenuated backbone were made.

The same phenotypic markers of attenuation were studied for each of the chimeras, including plaque size, temperature sensitivity, peak titers, and neurovirulence. Efficiency of growth, neutralizing antibody titers and immunogenicity were other experiments that were performed to compare the attenuation and immunogenicity of the chimeras to their wild type parental viruses. Results from the experiments to characterize and determine attenuation of each chimeric virus are shown in Figure 1.6 through 1.9 (*24*).

PLAQUE SIZE

A plaque assay was performed using the wild type viruses and each of the chimeras (Figure 1.6). The wild type viruses had the largest plaque sizes (see block bars in Figure 1.6). In comparison to the wild type, the chimeric viruses D2/1, D2/3, D2/4 produced smaller plaques. The chimeras containing the attenuated PDK53 backbone, either E or V

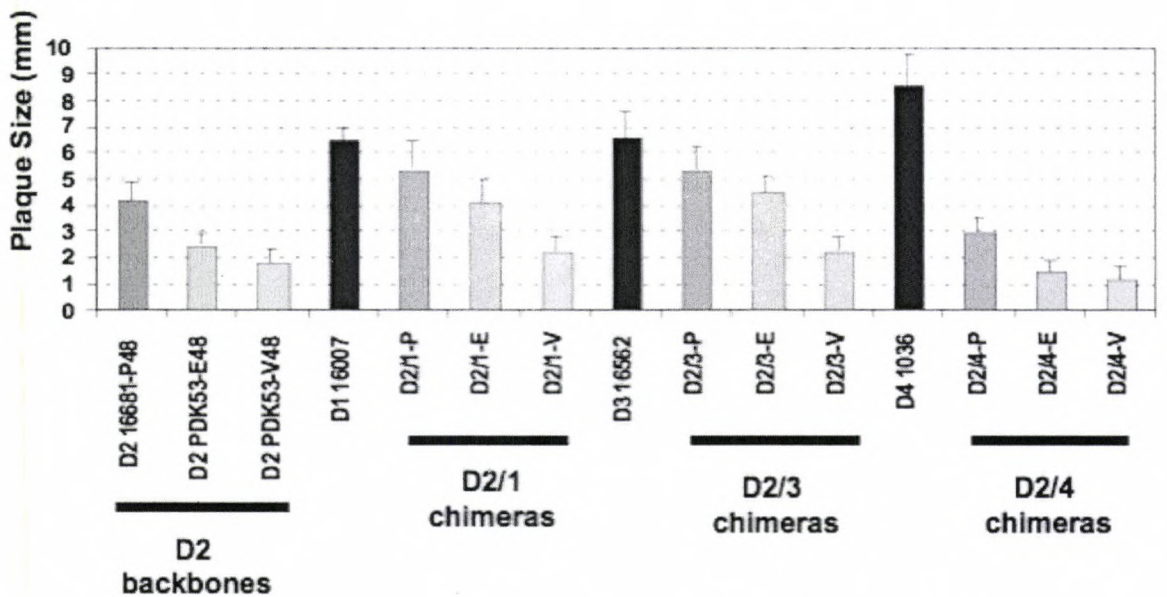

Figure 1.6 **Growth characteristics of chimeric dengue viruses in LLC-MK2 cells**. Mean (+/-) standard deviation plaque diameters. Values were calculated from 10 individual plaques of each virus on day 10 after infection. The graph bar heights represent the average plaque size in mm. Grey bars, D2 16681-P48 virus and chimeras containing that background, blue bars, viruses containing theD2 PDK53-E48-background; orange bars, D2 PDK53-V48 virus and chimeras with that background; black bars, wild-type D1, D3, and D4 viruses. P48 is the wild type dengue 2 parental backbone. E48 and V48 are the two different PDK-53 attenuated dengue 2 backbones containing the parental E (glutamate) at NS3-250 or attenuated V (valine) at NS3-250, respectively. Huang, C. Y. (2003) Dengue 2 PDK-53 virus as a chimeric carrier for tetravalent dengue vaccine development, *J Virol 77*, 11436-11447.

variants had the smallest plaque size. The small plaque size of the PDK53 chimeras suggests greater attenuation compared to the wild type virus.

Temperature Sensitivity and Peak Titers

Each of the chimeras was tested for temperature sensitivity in LLC-MK_2 cells (Rhesus monkey kidney) and Vero cells (kidney epithelial cells of African green monkey). A virus was considered temperature sensitive when the titer was reduced by greater than 90% when grown at 38.7°C compared to 37°C. The peak titers were also determined after growth in C6/36 cells (*Aedes albopictus* mosquito) and LLC-MK_2 cells. Figure 1.7 shows the results of the temperature sensitivity test as well as the peak titer test both using the LLC-MK_2 cells; results in other cell types are not shown. The wild-type viruses DEN-1, DEN-3 and DEN-4 were not temperature sensitive, indicated by a minus (-). The chimeras for each serotype that were constructed with the 16681 backbone had only marginal temperature sensitivity. The chimeras containing the attenuated PDK53-E and –V backbones were all temperature sensitive, indicated by a plus (+). The peak titers are shown in the bar graph of Figure 1.7. The lowest peak titers within each serotype specific group were seen with the PDK53-V backbone. The D2/1 and D2/3 chimeras in the DEN-2 16681 (P) backbone or PDK53-E backbone had peak titers comparable to their parental wild-type viruses. On the other hand, the D2/4 chimeras had much lower peak titers when cloned into the DEN-2 16681 (P) backbone or PDK53-E backbone when compared to the parental wild-type DEN-4 1036. The efficiency of replication was also tested in Vero cells, with the same results (data not shown). The chimeric viruses containing the DEN-2 PDK-53 backbone were shown to be more attenuated than the

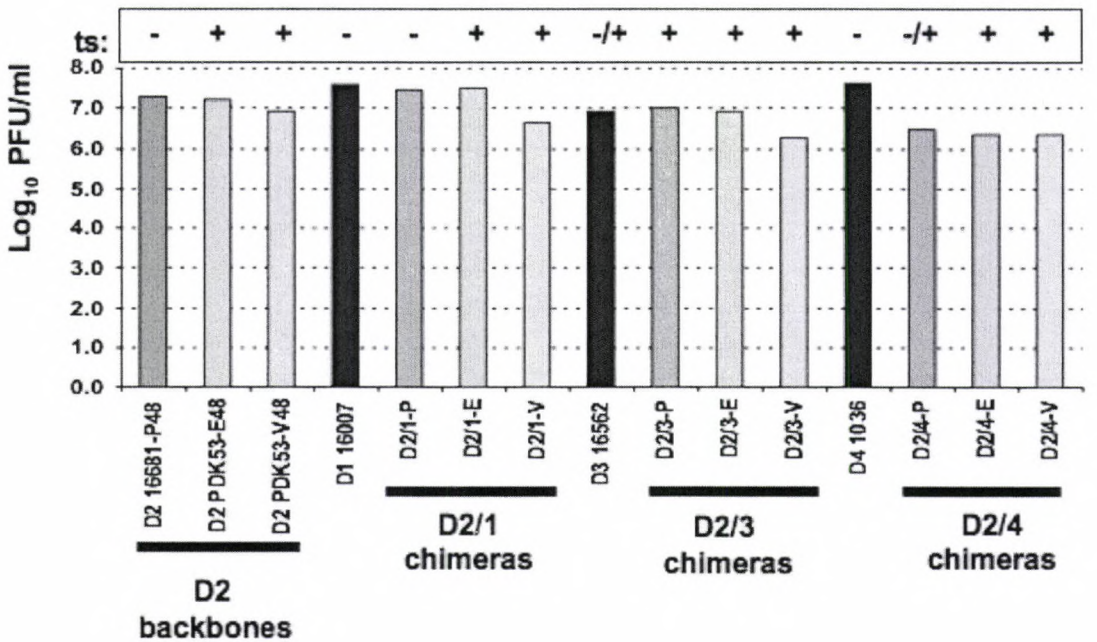

Figure 1.7 **Growth characteristics of chimeric dengue viruses in LLC-MK2 cells.** Temperature sensitivity (ts) and peak titers of viruses on day 6, 8, or 10 after infection. The ts scores were based on the reduction of the virus titers at 38.7°C versus the titers at 37°C (+ indicates titer reduction of 90% or greater at 38.7°C; +/- indicates reduction in the range that crosses the 90% cut off point from multiple experiments). Grey bars, D2 16681-P48 virus and chimeras containing that background, blue bars, viruses containing theD2 PDK53-E48-background; orange bars, D2 PDK53-V48 virus and chimeras with that background; black bars, wild-type D1, D3, and D4 viruses. Huang, C. Y. (2003) Dengue 2 PDK-53 virus as a chimeric carrier for tetravalent dengue vaccine development, *J Virol 77*, 11436-11447.

parental wild type viruses due to the increased temperature sensitivity and slightly decreased peak titers.

Efficiency of Replication in Mosquito Cells

The efficiency of replication was tested in C6/36 cells after 12 days of virus growth (*24*). The attenuated PDK53-E and V viruses had much lower titers as compared with the DEN-2 16681 parental virus, shown in the D2 backbones (Figure 1.8). Each of the wild type parental viruses had the highest peak titers. The chimeras containing the attenuated PDK-53 backbones had the lowest titers. The attenuated PDK53-E virus had higher efficiency of growth than either of the chimeras with the PDK53-E backbone, D2/3-E and D2/4-E. In contrast, the D2/3-V and D2/4-V chimeras replicated with approximately equal efficiency as the PDK53-V backbone. The D2/1 chimeras replicated differently than the D2/3 and D2/4 chimeras. Both the D2/1-E and D2/1-V chimeras had 10-fold less efficiency of growth compared with the PDK53-E and PDK53-V, respectively. Within the D2/1 chimeras the D2/1-V again had 10-fold lower replication efficiency than the D2/1-E chimera (*24*). The wild type viruses had much greater replication efficiency in C6/36 cells than the chimeric viruses containing the DEN-2 PDK-53 backbone, which shows that the chimeric viruses with the DEN-2 PDK-53 backbone are more attenuated. The replication efficiency of the viruses is important because the goal of vaccine manufacture and development is to make safe, highly replicating (*in vitro*) and stable viruses.

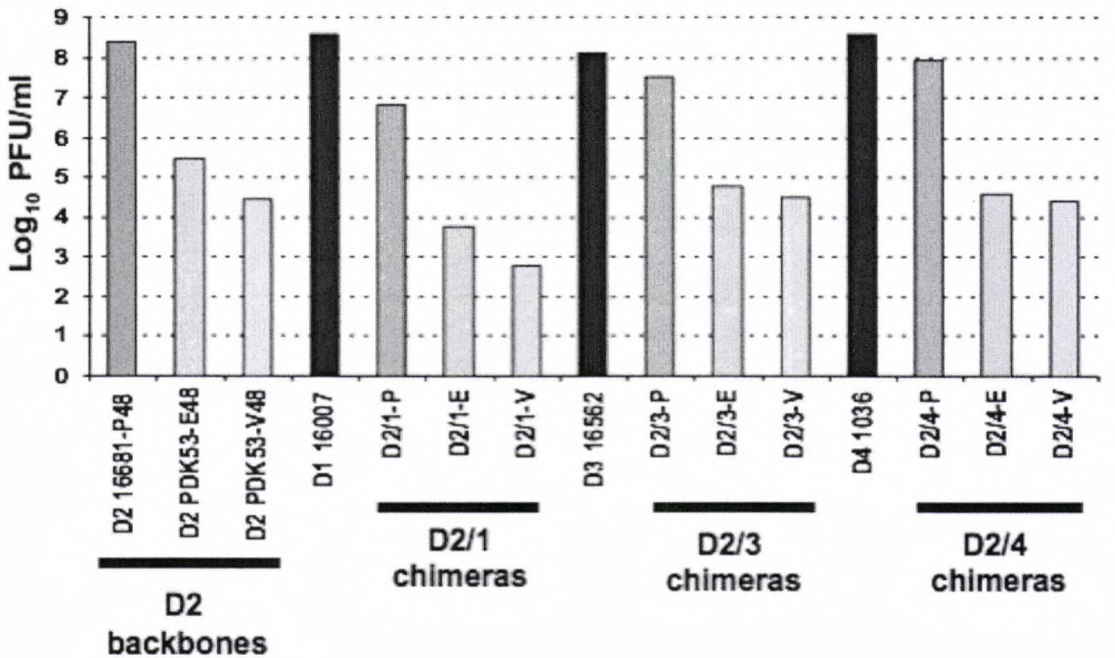

Figure 1.8 **Growth characteristics of chimeras in C6/36 cells.** Peak titers of viruses in C6/36 cells within 12-day cultures. Grey bars, D2 16681-P48 virus and chimeras containing that background, blue bars, viruses containing theD2 PDK53-E48-background; orange bars, D2 PDK53-V48 virus and chimeras with that background; black bars, wild-type D1, D3, and D4 viruses. Huang, C. Y. (2003) Dengue 2 PDK-53 virus as a chimeric carrier for tetravalent dengue vaccine development, *J Virol 77*, 11436-11447.

Neurovirulence of Chimeric Viruses

The chimeras were tested for neurovirulence in litters of newborn ICR mice by intracranial (IC) inoculation. The mice were observed for 5 weeks on a daily basis for paralysis or death (Figure 1.9). The DEN-3 16562 and DEN-4 1036 parental strains had 100% mortality, and the DEN-2 16681-P48 parental virus had 87.5% mortality. The D2/3-P and D2/4-P chimeras, containing the parental 16681 backbone had lower percentage of mortality as compared to the parental backbones, but still had 32.5% and 62.5% mortality, respectively. The D2/3 and D2/4 chimeras containing the PDK53-E or PDK53-V backbones and the DEN-2 PDK-53-E and -V backbones alone induced no mortality upon IC administration to newborn ICR mice (*24*).

Immunogenicity of Chimeras in AG129 Mice

A major hurdle for dengue vaccine development was finding a small animal model that is susceptible to challenge by wild-type virus. The dengue virus is not pathogenic in mice, which are the species commonly used as a small animal model. A novel small animal mouse model was used to assess vaccine safety, immunogenicity and efficacy. It was previously shown that certain interferons might play a part in dengue infection in humans (*25, 26*). The specific interferons associated with dengue infection are alpha and beta (IFN-α/β) and gamma IFN (IFN-γ). Groups of wild type mice (WT129) and mice that were lacking the IFN-α/β and IFN-γ receptor genes (AG129) were inoculated intraperitoneally with a mouse-adapted DEN-2 virus strain, Mochizuki, at 10^6 PFU. The AG129 mice all showed signs of neurological abnormalities and by day 12 were all dead.

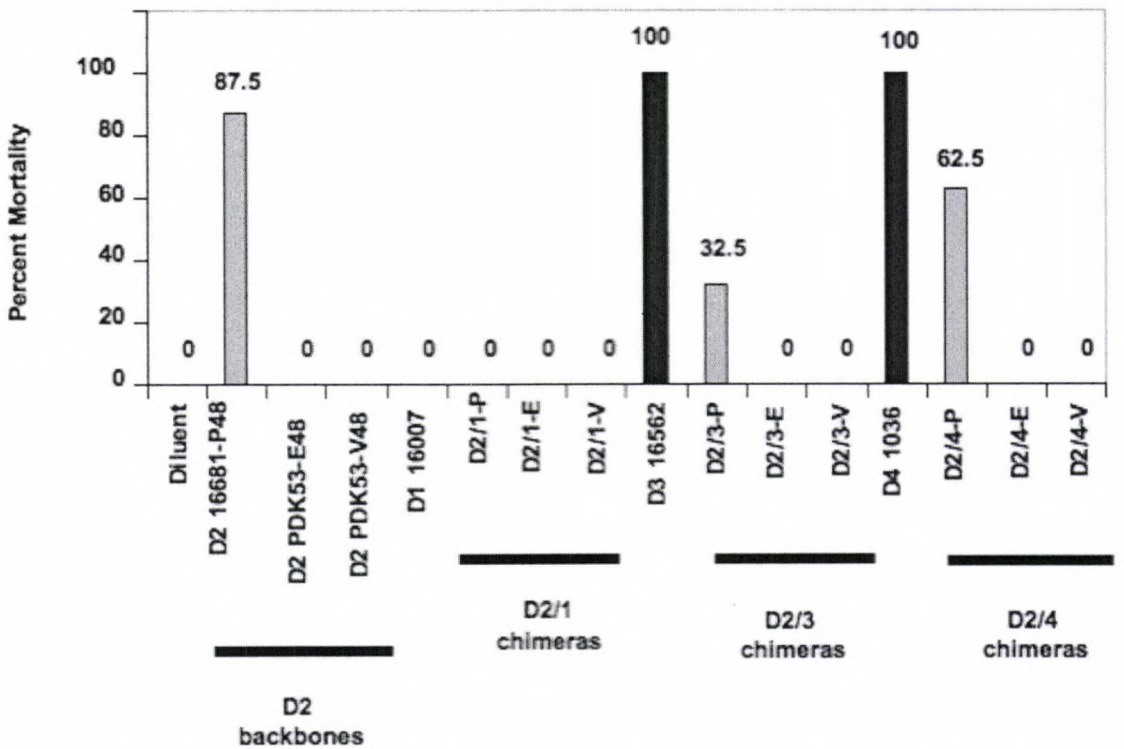

Figure 1.9 **Neurovirulence of chimeric D2/3 and D2/4 in newborn mice.** New born ICR mice were inoculated with 10^4 PFU of virus by the intracranial route. Percent mortality is indicated directly over each graph bar. Grey bars, D2 16681-P48 virus and the chimeras within that background; black bars, wild-type D3 and D4 viruses. Average survival times of D2 16681-P48, D3 16562, D2/3-P, D4 1036, and D2/4-P viruses were 15.6 ± 2.6, 14.1 ± 2.1, 19 ± 2.1, 8.6 ± 0.6, and 17.8 ± 2.8 days, respectively. Huang, C. Y. (2003) Dengue 2 PDK-53 virus as a chimeric carrier for tetravalent dengue vaccine development, *J Virol 77*, 11436-11447.

The WT129 mice showed no symptoms after injection with the mouse-adapted DEN-2 virus strain (*27*). The AG129 mice were then immunized with DEN-2 16681, the wild type parental strain, and DEN-2 PDK-53 attenuated strain to compare the neutralizing antibodies produced. The mice injected with DEN-2 16681 produced much higher neutralizing antibodies, which is comparable with previous data showing DEN-2 16681 replicates more efficiently than DEN-2 PDK-53 in mice. The AG129 mouse was accepted as an effective small animal model for dengue virus infection (*27*).

Further testing of chimeric vaccines in AG129 mice was performed. The AG129 mice were immunized with DEN-1 16007 or the D2/1 chimeras and then challenged with a lethal D1 Mochizuki (mouse-adapted) virus. The Mochizuki virus strain was previously shown to be lethal for adult AG129 mice (*24*). The mice immunized with DEN-1 16007 and the D2/1 chimeric virus survived the challenge. The control mice were immunized with a saline solution and died between 6 and 15 days after challenge. The mice that did survive had high neutralizing antibody titers to the wild type DEN-1 16007 virus. The D2/3 and D2/4 chimeras were also tested for immunogenicity in AG129 mice. Each of the chimeras tested were proven to be immunogenic in the mouse model. There was no challenge after immunization with the D2/3 and D2/4 chimeras because there are no DEN-3 and DEN-4 strains that are lethal to AG129 mice (*24*).

Tetravalent virus formulations were also tested for immunogenicity in AG129 mice (*24*). The mice were immunized with two different formulations, each containing all four serotypes. The first, called tetra-E, was composed of chimeras that were constructed with the PDK53-E backbone (D2/1-E, D2 PDK53-E48, D2/3-E, and D2/4-E). The second

formulation called tetra-V was composed of chimeras that were constructed with the PDK53-V backbone (D2/1-V, D2 PDK53-V, D2/3-V, and D2/4-V). After primary immunization, both formulations elicited similar neutralizing antibody titers against all four DEN serotypes. The tetravalent formulation containing the chimeric viruses and the monovalent vaccines elicited similar neutralizing antibody titers against DEN-1, DEN-3 or DEN-4 (*24*).

The PDK53-V variant was chosen as the backbone for each of the chimeric viruses. Figure 1.10 shows a schematic of the insertion of the wild type DEN-1, DEN-3 and DEN-4 structural genes into the PDK53-V nonstructural gene backbone. All four chimeric viruses have the DEN-2 PDK-53 backbone with the same three attenuating mutations and the prM and E structural genes specific to each separate serotype. The chimeric vaccine viruses are called DENVax-1 through DENVax-4.

1.10. Statement of Projects

Two separate projects will be discussed in this thesis involving the Dengue 2 PDK-53 based chimeric dengue vaccine. One of the projects aims to develop an assay to test for the residual host cell DNA in the dengue vaccine. The dengue vaccine is grown in an adherent cell line and the virus is clarified and harvested from the supernatant of the cell culture. When a virus infects cells, apoptosis can occur, which releases the cells DNA contents into the supernatant. The World Health Organization (WHO) and the US Food and Drug Administration (FDA) are concerned with the amount of host cell DNA that is

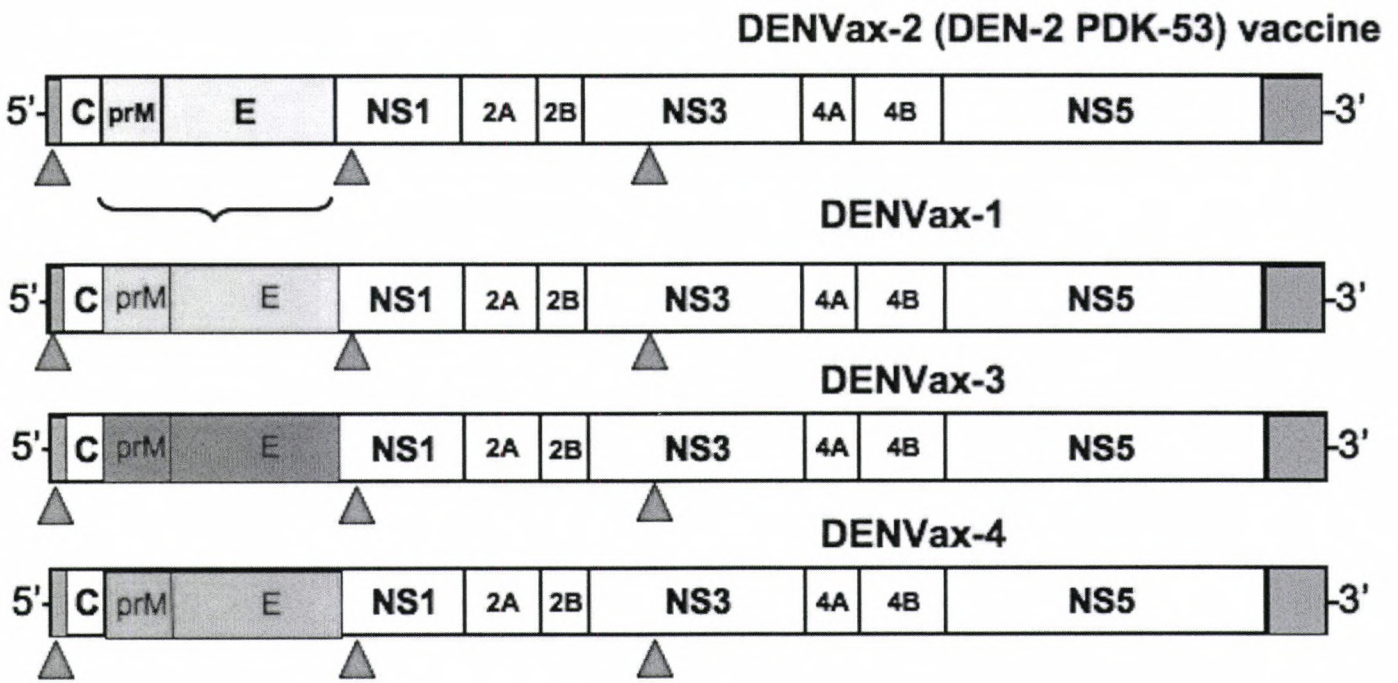

Figure 1.10 **Construction of each chimeric DENVax virus vaccine candidate.** The light blue prM/E sequence is dengue-2. The green prM/E sequence is dengue-1. The blue prM/E sequence is dengue-3. The orange prM/E sequence is dengue-4. Each chimera has the attenuated dengue-2 PDK-53 backbone with the corresponding prM/E sequence.

released into the supernatant, and consequently, harvested along with the virus. The WHO is concerned about the length and overall amount of residual host cell DNA that become incorporated into the vaccine. The concern is that with the use of a continuous/transformed cell line to make a vaccine, there is potential for the cellular DNA fragments to either integrate into the vaccine recipient's genome, or that the fragments are large enough to encode oncogenic activities. By developing an assay using quantitative real time polymerase chain reaction, we can accurately demonstrate the amount and size of the residual host cell DNA in the dengue virus vaccine samples.

The second project focuses on the production of a second-generation DENVax4 vaccine virus. The dengue vaccine is a tetravalent formulation containing attenuated viruses of each of the four serotypes. The immune response generated by the dengue 4 vaccine virus is limited, and therefore needs to be enhanced. The hypothesis is that by mutating the sequence 5' of the C/prM junction to be more like the dengue 4 wild type virus than the dengue 2 virus, the polyprotein will be more efficiently post-translationally processed. This will subsequently increase replication efficiency *in vitro* and *in vivo*. This increase in replication efficiency is hypothesized to produce more viral antigens and therefore the immunized individual will mount a greater immune response to the DENVax4 chimera. Three second generation candidate viruses were generated and compared to the original DENVax4 vaccine virus by genetic and phenotypic testing.

Both of these projects will aid in the development and efficacy of DENVax. The assay to quantify residual host cell DNA in the dengue vaccine, DENVax, will allow for accurate testing of the future manufactured lots. Also, the second generation DENVax4 has the

potential to produce a more robust immune response in animal models when compared to the original DENVax4. The second generation DENVax4 virus would be tested in the tetravalent formulation and possibly included in future clinical trials.

CHAPTER 2

DEVELOP A METHOD TO QUANTIFY HOST CELL (VERO) DNA LEVELS IN DENVAX

2.1. INTRODUCTION

Live attenuated virus vaccines are commonly grown *in vitro* on transformed mammalian cell lines. Vero cells are a continuous/transformed cell line and have deregulated the genes controlling cell growth, causing them to divide indefinitely. Throughout the course of vaccine manufacture host cells may die, leaving behind cellular debris, including DNA. The amount and size of residual host cell DNA in a virus vaccine when grown in a continuous cell line is a concern of the World Health Organization (WHO) and the Food and Drug Administration (FDA). The agencies are most concerned with larger DNA fragments; fragments smaller than 200 base pairs are less of a concern. The concern surrounding residual host/Vero cell DNA in human vaccines is the potential for the deregulated Vero genes to recombine into the vaccine recipients' genome, and lead to oncogenic or tumorigenic activity (*28*). Additionally, larger fragments of Vero DNA could possess one or more dominant activated oncogenes. There is also a fear that the DNA fragments could integrate into the host chromosomes and alter the normal

expression of the genes (*29*). If the residual DNA fragments in the vaccine sample are small enough, <200 base pairs, the likelihood that the fragments contain an activated oncogene is very low.

Inviragen's dengue vaccine viruses are grown *in vitro* on monolayers of African green monkey (*Cercopithecus aethiops*) kidney cells, also known as Vero cells. Vero cells are an adherent, continuous cell line and one of the few approved for human vaccine manufacture. Vero cells were derived from the kidney of an adult African green monkey (*Cercopithecus*) by Yasumura and Kawakita in 1963 (*30*). DENVax strains are grown and harvested from the supernatant of monolayers of infected Vero cells. Unlike many other viruses, dengue viruses do not induce apoptosis in their host cells and cause limited cytopathic effect (CPE). Because of this, the amount of residual DNA in the supernatant will be less than what is traditionally seen with other live attenuated virus vaccine types. However, dengue viruses are "slow growers" and require up to two weeks to reach peak titer. Therefore, the residual Vero cell DNA in the supernatant results from normal cell death after being grown in culture for two weeks without subculturing the cells.

In 1968, a WHO Study Group met to discuss cell substrates and their use for production of vaccines and other biologicals (*31*). They used an animal oncogene model to assess the risk of a cellular DNA transformation event. They determined that a transformation event would occur once in 10^9 recipients when exposed to 1 ng of cellular DNA that contained 100 copies of an activated oncogene (*32*). The conclusion of this meeting was that when using a continuous cell line, 100 picograms or less of residual DNA was a safe level per dose (*28*). Since then the WHO has determined that 10 ng or less residual DNA

in the vaccine is a safe level. The host cell DNA levels in a vaccine depend on the manufacturing process and the vaccine virus type (*29*). Inviragen has refined the manufacturing of the DENVax vaccine to limit the amount of residual host cell DNA. The virus manufacture protocol includes extensive washing and media replacement throughout the growth. The amount of Vero DNA left in the supernatant decreases at later harvest dates due to the washing of the cell monolayer following media change and virus harvest.

A quantitative real time PCR (qPCR) assay was developed to quantify and evaluate the host cell DNA levels in DENVax. Additionally, to evaluate the size of residual host cell DNA, primer sets were designed to amplify varying sizes of DNA fragments. This method allows total DNA quantification as well as DNA fragment sizes to be evaluated in the vaccine.

2.2. METHODS AVAILABLE FOR DNA QUANTITATION

We chose real time qPCR to evaluate host cell DNA levels in DENVax. Quantitative real time PCR is an extremely sensitive method for DNA quantitation within a sample. Real time qPCR eliminates the step after the reaction, as in regular PCR, when the DNA needs to be run on gel and quantified. At the end of a qPCR reaction, the data generated are analyzed by a program to give a standard curve, sample DNA amounts based on the standard curve, and efficiency of the run. There is also a melt curve generated that can show if there is more than one product being amplified. Although real time qPCR is a

sensitive and effective method to quantify DNA, the reaction conditions including primer design and annealing temperature need to be optimized for accurate data to be generated.

There are other methods available to detect and quantify DNA. One method for DNA quantification is nick translation. First, the DNA is briefly digested with DNase to produce single stranded "nicks" in the sequence. These DNA strand breaks have free 3' OH termini that can serve as templates for incorporation of radiolabeled deoxynucleotide triphosphates by *E. coli* DNA polymerase I (*33*). Nick translation is a good method for DNA quantification but it uses a radioactive nucleotide, which Inviragen does not have the capabilities to support.

Another alternative method for DNA quantification is a Southern blot. Our contract manufacturer for testing the residual Vero cell DNA in DENVax currently uses a Southern slot blot. Southern blotting is an effective method to approximate DNA amounts in a sample, but it is not nearly as quantitative as qPCR. The DNA is purified from the virus sample and digested with a restriction enzyme to cut the DNA into smaller fragments. It is then denatured and cross-linked to a nylon membrane. A fluorescent or radioactive probe is hybridized to the DNA and then visualized. The amount of DNA on a slot blot is visually estimated by comparing the samples to a DNA standard curve containing known amounts of Vero DNA. Since the WHO is looking for a specific amount and size of residual DNA, the slot blot method is not as accurate and quantitative as needed.

2.3. MATERIALS/METHODS

2.3A. DNA EXTRACTION/PURIFICATION

Vero DNA was extracted and purified from the Shantha Working Cell Bank (WCB) Vero DNA cell line (Passage 143) for the PCR standard curve. Shantha Biotechnics originally obtained the Vero cells from the European Collection of Cell Cultures (ECACC). This seed stock was at passage 134 (P-134) from the World Health Organization (WHO) Lot No. CB884. The passage number of Shantha's Working Cell Bank (WCB) is P-141.

The Vero cells were trypsinized, enumerated, and resuspended in PBS. The DNA was then purified from the cells using Qiagen's QIAamp DNA mini kit for DNA purification according to the manufacture instructions. The eluate from the Qiagen kit was treated with RNase A (Invitrogen, Carlsbad, CA). The DNA was further purified by standard phenol chloroform/isoamyl alcohol extraction. An equal volume of phenol chloroform isoamyl alcohol was added to the sample and vortexed for 30 seconds. The DNA was centrifuged in a microcentrifuge at 14K rpm for 5 minutes at room temperature. The top aqueous layer containing the DNA was removed from the lower organic layer containing the proteins. $1/10^{th}$ the volume of 5M NaOAc, pH 5.2 and 2.5x the volume of 100% ethanol (EtOH) were added to the aqueous phase. The mixture was inverted and stored at -20°C overnight. The next day the samples were centrifuged at 4°C, 16,000 x g for 20 minutes in a microcentrifuge. The EtOH was subsequently removed, and the DNA pellets left to air dry. Once dry, the pellets were resuspended in RNase free Tris-EDTA (TE) and stored at -20°C until use.

2.3B. RNase Treatment

The Vero standard DNA as well as the vaccine samples were RNase treated before being tested. 1 µl of RNase A (100 mg/ml; 7000 units/ml) (Qiagen, Valencia, CA) was added for every 50 µl of vaccine virus samples. The mixture was then incubated at 37°C for 30 minutes. The concentration was determined by spectrophotometry at OD_{260} and then stored at -20°C until use.

2.3C. Cloning

A ligation/transformation step was performed when generating the internal control samples to insert the internal control fragments into a pUC57 plasmid to grow. DH5α competent *E. coli* cells (Invitrogen) were placed on ice to thaw from -80°C for 15 minutes. While the cells were thawing the ligation reaction was set up using T4 DNA Ligase and a total reaction volume of 20 µl. The ligation reaction was performed at room temperature (20-25°C) for ten minutes. The competent cells were split into 50 µl aliquots per reaction and 2 µl of the ligation reaction was added to the 50µl of cells, followed by gentle mixing. The ligation reaction was incubated for 30 minutes on ice, followed by a 20 second heat shock at 42°C, and placed on ice for an additional 2 minutes. Luria Bertani (LB) medium was added and incubated on a shaker at 37°C for one hour. The samples were then plated on LB-agar plates with ampicilin and incubated at 37°C overnight. The next day colonies were picked and grown up in medium with ampicilin.

2.3D. iQ™ 5 Real-Time PCR Detection System

We used the Bio-Rad iQ™ 5 Real-Time PCR Detection System. The SYBR Green reactions use the Rio-Rad iQ SYBR Green Supermix, whereas the Taqman reactions use the iQ Supermix that can detect multiple probes.

Two methods are available to detect a PCR product using qPCR; SYBR Green and Taqman. SYBR green is a double stranded DNA binding dye (Figure 2.1). When it non-covalently binds to double-stranded DNA, the fluorescence emitted can be detected and is directly proportional to the amount of product amplified. The SYBR green dye will bind to all double stranded DNA being amplified in the reaction, even nonspecific products. It is a very effective way to measure total DNA being amplified and quantify the original DNA levels in a sample.

When using a Taqman assay for qPCR, specific fluorogenic probes are designed with the primer sets (Figure 2.2). The probes are sequence-specific and have a reporter and a quencher at either end of the oligo. The 5' exonuclease activity of the DNA Taq polymerase (TaqMan) cleaves the fluorophore from the probe and then knocks off the oligo as the DNA is being amplified. When the reporter is in close proximity to the quencher, it does not fluoresce. Once the reporter is cleaved due to the Taq polymerase, it is no longer close to the quencher and fluoresces. This florescence can be detected and is directly proportional to the amount of specific double stranded DNA being amplified in the sample. Taqman is a sequence-specific way to quantify the DNA levels in a sample.

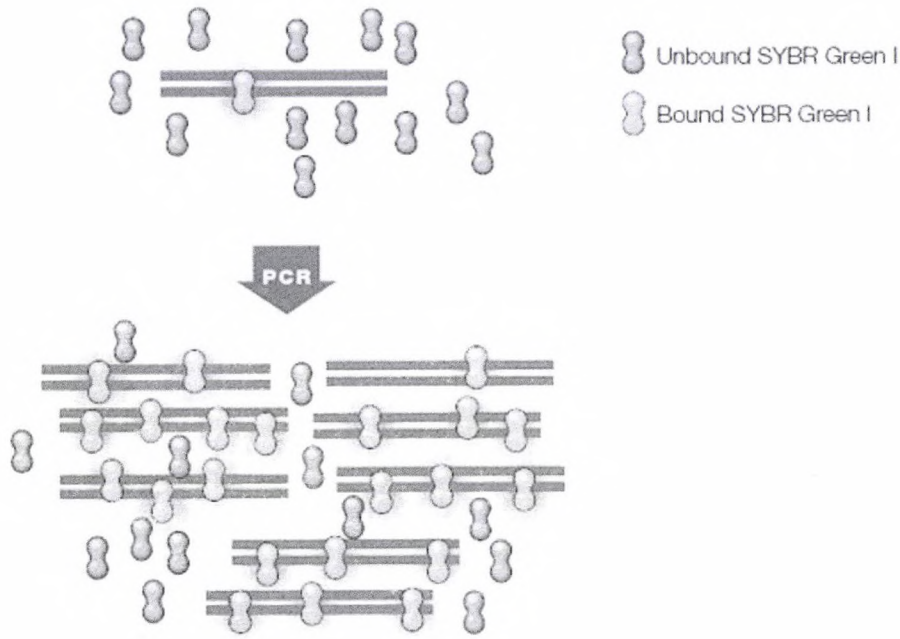

Figure 2.1 **SYBR Green detection for real time qPCR.** SYBR Green nonspecifically binds to double stranded polynucleotides. Upon binding, the amount of SYBR Green fluoresces is directly proportional to the amount of double stranded DNA product. This method quantifies all amplified products, including primer oligomers and nonspecific products. (Figure reproduced from Bio-Rad).

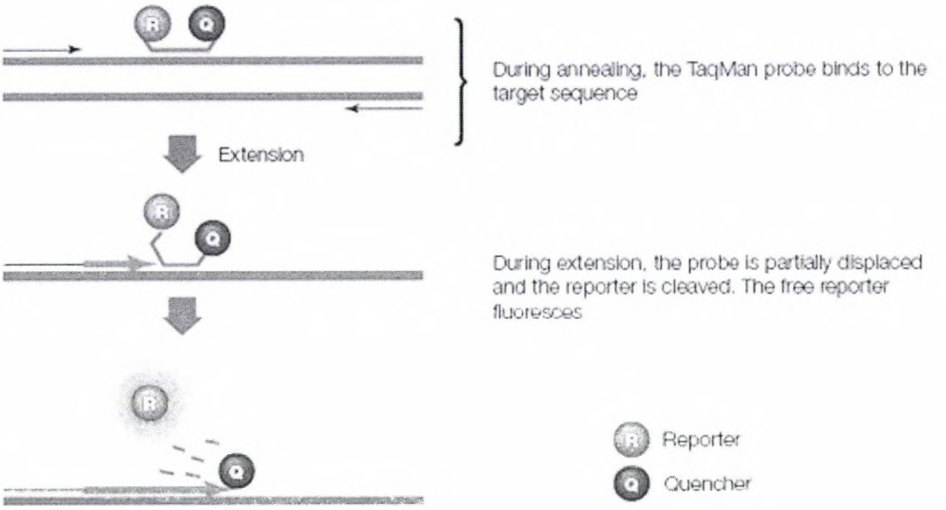

Figure 2.2 **Real time qPCR using TaqMan technology.** The Taqman method uses a sequence specific oligonucleotide probe which binds to the PCR product. The probe initially contains a reporter and quencher molecule at opposite ends which effectively limits its fluorescence until the quencher is cleaved. Taq DNA Polymerase contains 5' exonuclease activity which cleaves the fluorescent reporter. When the reporter is no longer in close proximity to the quencher, it fluoresces. This signal can be quantified and is directly proportional to the sequence specific product amount in the sample. (Figure reproduced from Bio-Rad).

2.3E. PRIMER DESIGN

The Alu Primer sets were designed using sequences from Pubmed accession number: DQ977866 *Chlorocebus aethiops* Alu insertion locus PM_PY2_48 genomic sequence. There are about 10^6 copies of Alu per human/primate genome. Alu elements are short interspersed nucleotide elements (SINEs) found in the noncoding regions of primate genomes. The Alu repeats are about 300 bp in length and contain a polymerase III promoter (*34*). The Alu recognition sequence is 5' AG/CT 3'. Alu Primer Set 1: 131 bp amplicon (Alu 1F: ACCATCCTGGCTA ACACGGTGAAA & Alu 1R:TTCCGGGT TTACGCCATTCTCCT). Alu Primer Set 2: 67 bp amplicon (Alu 2F: ACCATCCTGGCTAACACGGT GAAA & Alu 2R: CCTCGCCCGGCTAGTTT). Alu Primer Set 3: 219 bp amplicon (Alu 3F: CCTGTAA TCCCAGCACTTTGGGAG & Alu 3R: CGGATCTCAGCTCACTGCAAGCTC). Alu Primer Set 4: 481 bp amplicon (Alu 4F: ACCCTCATTTGAACAGGAACTCA & Alu 4R: GTACTCACATAATTCGAGT GCT). Alu Primer Set 5: 434 bp amplicon (Alu 5F: GGAGTTGAAGATTACCCTCATT TGAACAGG & Alu 5R: TTTGAGACGGAGTCTCGCT). Alu Primer Set 6: 425 bp amplicon (Alu 6F: GATTACCCTCATTTGAACAGGAACTCA & Alu 6R: TTTGAGA CGGAGTCTCGCT). Alu Primer Set 4F3R: 374 bp amplicon (Alu 4F: ACCCTCATTT GAACAGGAACTCA & Alu 3R: CGGATCTCAGCTCACTGCAAGCTC).

The 28S Ribosome Primers were designed based from the pubmed accession number: M27830 Human 28S ribosomal RNA gene, complete cds. Ribo Primer Set 1: 467 bp amplicon (Ribo56F: AGACCGTCGTGAGACAGGTTAGTT & Ribo499R: TCTACGA ATGGTTTAGCGCCAGGT). Ribo Primer Set 2: 235 bp amplicon (Ribo9F: CAAGCG

TTGGATTGTTCACCCACT & Ribo220R: AGGCGTTCAGTCATAATCCCACAG). Ribo Primer Set 3: 119 bp amplicon (Ribo56F: AGACCGTCGTGAGACAGGTTAGTT & Ribo151R: AATGTCTGAACCTGCGGTTCCTCT).

Histone H4 Primers were designed based from the pubmed accession number: X60481 *H. sapiens* H4/a gene for H4 Histone. Histone Primer Set 1: 422 bp amplicon (H4-77F: TT TCAGTCTCTGTGTCCG CTGGAG & H4-498R: GCAACCTTAACCGCCAAAGC CATA). Histone Primer Set 2: 92 bp amplicon (H4-241F: AAGGTGTTGCGTGACAA CATCCAG & H4-350R: ATCAGACCAGAGATCCGCTTCACA).

Actin Primers were designed based from the pubmed accession number: AB004047 *Cercopithecus aethiops* mRNA for beta-actin, complete cds. There are 20 actin repeats per human diploid genome. Actin Primer Set 1: 103 bp amplicon (A1-F857: ATGAA GTGTGACGTGGACATCCGT & A1-R959: TCTCCTTCTGCATCCTGTCAGCAA). Actin Primer Set 2: 217 bp amplicon (A2-F816: TGGAGTCCTGTGGCATCCATGAAA & A2-R1032: ATCCACACAGAGTACTTGCGCTCAG). Actin Primer Set 3: 409 bp amplicon (A3-F624: AGCGGGAAATCGTGCGTGACATTA & A3-R1032: ATCCAC ACAGAGTACTTGCGCTCAG). Actin Primer Set 4: 401 bp amplicon (A4-F191: AAG AGAGGCATCCTCAC CCTGAAGTA & A4-R591: GTGAGGATCTTCATGAGGTAG TCAGTCAGG). Actin Primer Set 5: 359 bp amplicon (A3-F624: AGCGGGAAATCGTGCGTGACATTA & A1-R959: TCTCCTTCTGCATCCTGTCAG CAA). Actin Primer Set 6: 441 bp amplicon (A6-646F: TAAGGAGAAGCTGTGCTAT GTCGC & A6-1086R: TTGCTGATCCACATCT GCTGGAAG). Actin Primer Set 7: 354 bp amplicon (A7-784F: TGAGGCTCTCTTCCAACCTTCCTT & A7-1137R: TA

GAAGCATTTGCGGTGGACGATG). Actin Primer Set 8: 407 bp amplicon (A8-241F: CAACTGGGACGACATGGAGAAGAT & A8-647R: TAATGTCACGCACGATTTCC CGCT).

2.3F. VERO DNA STANDARD CURVE

The standard curve was generated by extracting Vero DNA using a Qiagen kit, followed by a standard phenol-chloroform extraction, and RNase treatment (see section 2.3A). The concentration was quantified by spectrophotometry and stored at a 100ng/μl stock solution. To generate a standard curve, serial dilutions in water were performed before each experiment from 100 ng to 0.001 ng. The WHO is concerned about DNA levels exceeding 10 ng/dose, so we set our experimental detection limit in the standard curve at 0.001 ng.

2.3G. GENERATION OF INTERNAL CONTROL SAMPLES

Sequence from the F1 protein of the gram-negative bacteria, *Yersinia pestis*, was used as filler DNA (*35*). A plasmid containing the F1 coding region of *Yersinia pestis* was obtained from USAMRIID by Heska and then cloned into a Raccoon pox transfer vector. Inviragen subsequently signed a Material Transfer Agreement (MTA) with Heska to obtain the plasmid containing the F1 sequence. Primer sets were designed for each of the four internal control fragments being generated, that had a HindIII site at the end, followed by the Alu or Actin Vero primer sequences, and then the F1 sequence (Figure 2.3). PCR was performed to amplify the internal control DNA containing the correct primer sets flanking the F1 sequence. Phusion Hot Start DNA polymerase was used with

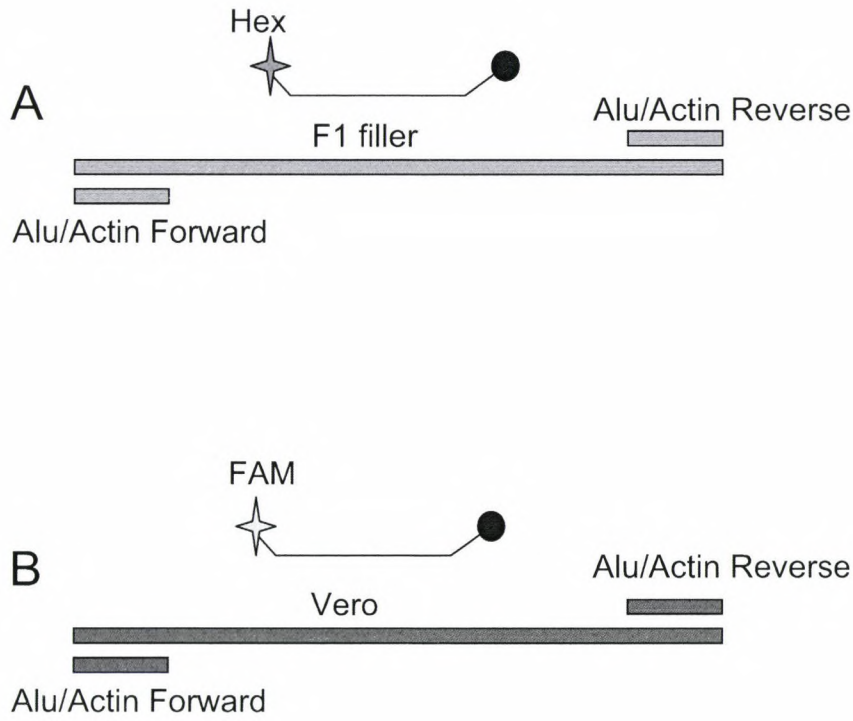

Figure 2.3 **Generation of internal control samples.** The internal controls are amplified by the same primer set desiged to amplify the Vero DNA target, and are the same size as the amplicon (Alu 1-IC: 132 bp, Actin 1-IC: 103 bp, Actin 3-IC: 409 bp). To allow duplexing, we designed the two TaqMan probes to contain different fluorescent reporters.

the F1 DNA as the template, 5x HF Buffer, and 10mM dNTPs. Once the amplicon was generated, the samples were Geneclean® purified to isolate the DNA from the enzymes and other components of the PCR reaction mix. The internal control DNA was then HindIII digested to isolate the fragment of interest from the rest of the F1 input DNA sequence. The vector pUC19, was also HindIII digested and then phosphatase treated using Antarctic Phosphatase to prevent re-annealing of the sticky ends before the internal control DNA could be ligated into the plasmid (Figure 2.4).

The pUC19 vector and the internal control DNA (one experiment for each of the 3 sizes and primer set internal control samples) were ligated using T4 DNA Ligase. The reaction was transformed into competent DH5α *E. coli* cells and then grown on LB agar/ampicillin plates. Three colonies from each plate were chosen and amplified in LB/ampicilin medium the DNA samples were purified with a Qiagen miniprep kit. Once amplified and purified, the internal control samples were again HindIII digested and gel purified from the pUC19 backbone. The same F1 filler DNA was used for each of the internal control samples. A probe was designed for the F1 filler DNA internal control samples using the reporter molecule HEX. HEX is excited at 535 nm and emits light green at 556 nm. Two different probes were designed for the Vero DNA amplicons, Alu and Actin using the reporter molecule FAM (Figure 2.3). FAM is excited at 495 nm and emits dark green at 516 nm. These are two compatible fluorophores that are commonly used together in duplexing qPCR reactions.

The concentration of the internal control samples, called Alu1-IC, Actin1-IC, Actin3-IC, was determined using spectrophotometry at OD_{260}. A TaqMan protocol for qPCR was

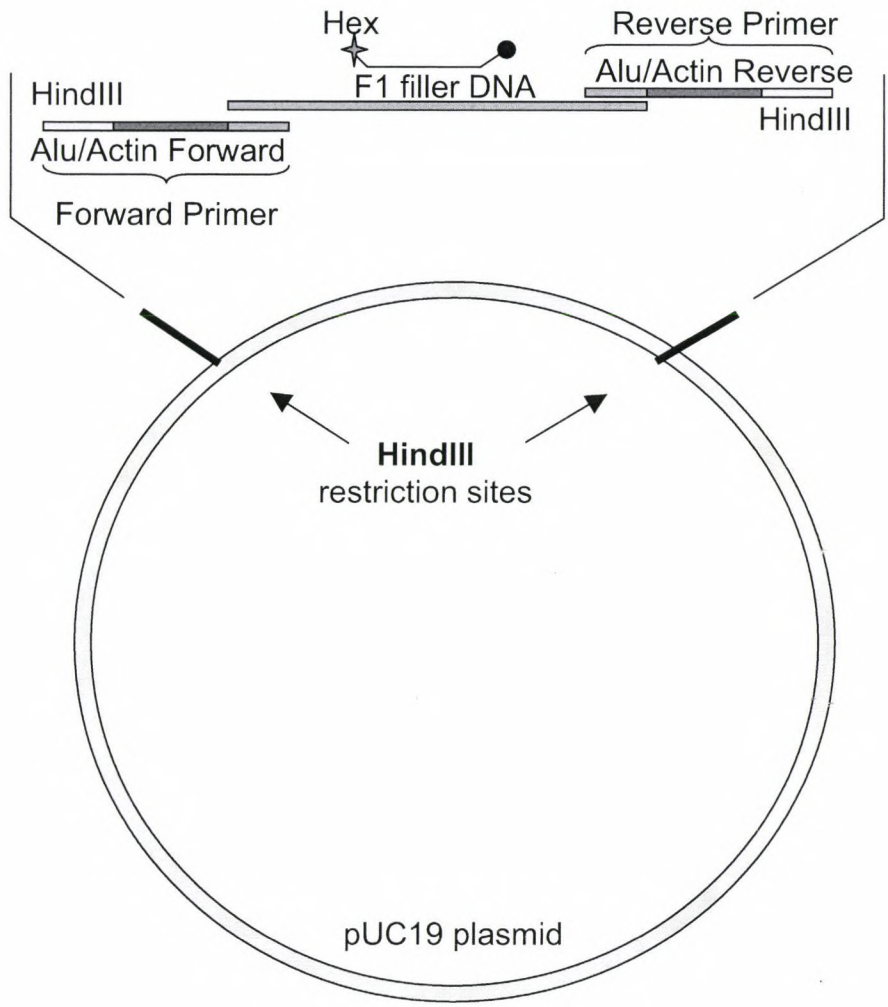

Figure 2.4 **Internal control design.** The internal control DNA oligonucleotides were cloned into a pUC19 vector. Primers were designed which contained either Alu or Actin primer sequences flanked by HindIII digestion sites and an appropriate length of F1 sequence to amplify our nonspecific "filler" DNA. The fluorescent TaqMan probe was designed to bind the internal control DNA sequence, with a Hex reporter molecule for detection.

designed using specific primers designed to anneal to the internal control DNA or the Vero DNA. A FAM probe was designed for the Vero Alu sequence that would anneal to the amplicons generated when using the primer set Alu1. A different FAM probe was designed for the Vero Actin sequence that would anneal to the amplicons generated when using the primer sets Actin1 and Actin 3. They were both FAM because they would never be used in the same reaction. A HEX probe was designed for the internal control F1 DNA, which anneals to all of the internal control sequences.

2.4. Results

2.4a. Sample preparation

After analysis of our original Vero DNA purification, it was discovered that some of the polynucleotides being detected during UV spectrometry were a result of co-purified RNA. We subsequently treated these samples with RNase and obtained a pure sample of Vero DNA. Two separate serial dilutions were generated to test in qPCR; one with untreated Vero DNA, a second with RNase treated Vero DNA. Typical standard curves of the Vero DNA before and after RNase treatment were evaluated as shown in Table 2.5. This data showed that there was RNA present in the standard Vero DNA and therefore the concentration was incorrect. We therefore RNase treated the vaccine virus samples before DNA detection by qPCR.

A

Vero DNA Standard Before RNase Treatment	
C_T	ng
7.64	100
11.07	10
14.74	1
18.29	0.1
21.77	0.01
25.33	0.001

B

Vero DNA Standard After RNase Treatment	
C_T	ng
4.87	100
8.64	10
12.21	1
15.97	0.1
19.72	0.01
23.59	0.001

Table 2.5 **RNA contamination of standard curve Vero DNA.** The standard curve Vero DNA was RNase treated by adding 1 μl of RNase A (100mg/ml) to a 50 μl DNA sample, and incubating for 30 minutes at 37°C. Serial 10-fold dilutions were generated containing 2 μl DNA and 18 μl water. (A) shows the standard curve of the Vero DNA before RNase treatment. (B) is the standard curve of Vero DNA after RNase treatment. The C_T is the threshold cycle.

When first considering real time qPCR as an option for quantifying Vero cell DNA amounts, we discovered that the cell culture medium the vaccine virus is grown and stored in was interfering with the PCR reaction. Representative PCR reactions were performed, testing varying media dilutions and types (Table 2.6). Serial ten fold dilutions were generated using RNase/DNase free water to a dilution of 1:100,000. The three different media tested were DMEM with 0.1% F127 (a block copolymer surfactant), TFA (15% Trehalose, 1% F127, 0.1% Human Serum Albumin-HSA), and DMEM with 0.1% F127 and 10% Fetal Bovine Serum (FBS). A known amount (0.1 nanogram) of Vero DNA was added to each sample. This amount of DNA was detected at a C_T of 16.7 in the standard curve. Without any dilution, all media showed complete interference with the PCR reaction and no PCR product was detected (Table 2.6, Dilution of 1), shown as not available (N/A). At the 1:10 dilution there was still interference of the media because the C_T and ng values are not the expected values. The 1:100 and 1:1000 dilutions were both dilute enough to eliminate the medium interference as shown in Table 2.6, and the 1:1000 dilution was chosen for further experiments. After testing vaccine virus samples at a 1:1000 dilution, we confirmed that the assay was sensitive enough to detect the DNA levels within the standard curve, 100 ng- 0.001ng (data not shown). Before each experiment, the samples were diluted to 1:1000 in RNase/DNase free water and tested. When using the dilution method for sample preparation, the vaccine virus samples were tested using SYBR green reaction mix by Bio-Rad.

Preparing the samples by diluting 1:1000 in water is attractive because there is no loss of sample after extraction, as seen when using a Qiagen column to purify the Vero DNA. By diluting, the entire sample is retained and an accurate amount of DNA can be detected

Medium Dilution	DMEM 0.1% F127		TFA		DMEM 0.1%F127 10%FBS	
	C_T	total ng	C_T	total ng	C_T	total ng
1	N/A	N/A	N/A	N/A	N/A	N/A
1:10	16.76	0.103	16.55	0.116	18.59	0.0352
1:100	16.17	0.146	16.21	0.142	16.11	0.151
1:1000	15.99	0.162	16.33	0.133	16.08	0.154
1:10000	16.17	0.146	16.37	0.13	16.53	0.119
1:100000	16.12	0.15	16.35	0.131	16.28	0.137

Table 2.6 **Effect of medium on qPCR.** Three different types of vaccine storage medium were diluted in water. A known amount of Vero DNA (0.1 ng) was added to each sample and quantified by qPCR after serial dilution. The standard curve C_T for 0.1 ng of DNA came up at 16.7. The purpose was to test the dilution at which the medium no longer interferes with the reaction. F127 is a block copolymer surfactant. TFA is 15% Trehalose, 1% F127, 0.1% HSA. Fetal bovine serum (FBS).

from PCR. The disadvantage to diluting the samples is that the Actin primers (compared to the Alu primers) may be insufficiently sensitive to detect the original amount of Vero DNA in samples.

The second method we investigated to prepare the vaccine virus samples was to extract the DNA using a Qiagen column. The samples were RNase treated prior to DNA extraction. We performed many replicate extractions containing a known amount of DNA spiked into the sample to estimate how much was recovered during column purification using the SYBR Green method of qPCR. The goal was to calculate a standard percent recovery to apply to all vaccine virus samples when extracted using Qiagen's column (data not shown). Additionally, it was found that the length of DNA recovered after extraction also varied. The binding efficiency of the column varies depending on the size of the DNA fragments that are being purified. Since we are testing for DNA fragments of different sizes, the percent recoveries varied for each primer set being used during quantification (data not shown). There was no reproducible trend in recoveries when comparing sizes ranging from 100 bp to 400 bp, showing that the recovery of DNA was dependent on fragment size.

2.4B. Design multiple primer sets to quantify DNA fragment sizes for qPCR using SYBR Green

Multiple primer sets were designed to amplify different sequences from the African green monkey genome, including Alu repeats, actin, ribosome, and histone sequences. Alu

elements were originally identified as regions containing recognition sites for the restriction enzyme AluI. Full-length Alu elements are about 300 bp in length and are the most abundant mobile elements in the human/primate genome, containing about 10^6 copies per diploid genome (*36*). These Alu elements are repeated multiple times in the genome but are not uniformly distributed. The high copy number of the Alu repeats make it suitable to detect a small (10 ng) amount of host cell DNA in a vaccine sample. A high copy number gene may provide the sensitivity required to enumerate this small an amount of DNA. The Alu 1 primer set 1 design was based on the Alu repeat sequence in Vero cells (Figure 2.7). Although the Alu sequences are highly repetitive, we found that the Alu primers were able to detect even the slightest contamination in the no template control (NTC) samples, discussed later.

The number of ribosomal RNA gene repeats can vary from 300 to 600 copies per cell depending on the species. Due to the high copy number in humans/primates, primers were designed based on the 28S ribosomal RNA. The ribosomal RNA gene sequences in *Chlorocebus aethiops* have not been published, so the primers were designed from the human 28S ribosomal RNA gene sequence. These primers were not sensitive enough to detect 10 ng/dose that we need for the WHO standards (data not shown).

The histone H4 primer sets were then tested. The histone primer sets, like the ribosomal RNA primers, were not sensitive enough to detect the 10 ng of Vero DNA per vaccine dose (data not shown).

LOCUS DQ977866 545 bp DNA linear PRI 20-FEB-2007
DEFINITION C hlorocebus aethiops Alu insertion locus PM_PY2_48 genomic sequence.

```
1   attagagact tctgatttaa aatggagttg aagattaccc tcatttgaac aggaactcaa
61  aagttggcaa catagcataa aatctgtacc agacctctaa gtttcttgtt attgctgttg
121 tctttcatgt gataggaatc tggttggggt tttaaaaata catagttatt ggccgggcgc
181 ggtggctcaa gcctgtaatc ccagcacttt gggaggccga gacgggagga tgacggggtc
241 agcagatcga gaccatcctg gctaacacgg tgaaaccccg tctctactaa aaaaaataca
301 aaaaactagc cgggcgaggt ggcgggcgcc tgtagtccca gctactcggg aggctgaggc
361 aggagaatgg cgtaaacccg gaaggcggag cttgcagtga gctgagatcc ggccactgca
421 ctccagcctg ggcgacagag cgagactccg tctcaaaaaa aaaaaaaaaa aaaaaaatac
481 atagttatta ttttaaagca ctcgaattat gtgagtactt aataccattt gatgtcatta
541 gatac
```

Alu Primer Set 1: 132 bp amplicon

Figure 2.7 **Alu1 primer set design.** The Alu 1 primer set, with a 132 bp amplicon, was designed using the *Chlorocebus aethiops* Alu insertion genomic sequence.

The actin primer sets we moved forward with are shown in Figure 2.8. The larger primer set, Actin 3 (409 bp) flanks the smaller primer set, Actin 1 (103 bp). There are about 20 actin gene repeats in the genome of humans/primates (*37*). It is not a high copy number gene but it is much more predictable than the randomly dispersed Alu gene repeats. The actin genes have been split into three main groups the α, β, or γ actin genes, which can be separated by isoelectric focusing (*38*). Although there are different groups of actin genes, there is >90% homology in their amino acid sequences (*39*). The β and γ actins are mostly cytoplasmic and are involved in cytoskeleton structure and cell motility. The α actins are located in muscle tissues and involved in contraction.

2.4C. CREATE INTERNAL CONTROLS TO EVALUATE DNA YIELD AFTER QIAGEN COLUMN PURIFICATION

The data generated by the previously described experiments when samples were spiked with a known concentration of Vero DNA were extracted and a percent loss was calculated. This experiment demonstrated that yields during purification were inconsistent and not statistically significant enough to apply to all samples. To control for the percent loss on the Qiagen column, internal control samples were created that could be co-purified over a Qiagen DNA purification column alongside the vaccine samples. A known quantity of the internal control DNA was added, and then tested by real time PCR to obtain a percent loss value. The percent loss value would then be applied to the vaccine samples to estimate total DNA. We created synthetic DNA oligonucleotides flanked by sequences which would anneal to the same primers for amplification as the Vero DNA fragments, and are about the same size as the amplicons

Actin Primer Sets:
Cercopithecus aethiops mRNA for beta-actin, complete cds
Accession: AB004047

1138 bp

```
   1 aactcacgtt atggatgatg atatcgccgc gctcgttgtc gacaacggct ccggcatgtg
  61 caaggccggc ttcgcgggcg acgatgcccc tcgggccgtc ttcccctcca tcgtggggcg
 121 ccccaggcac cagggcgtga tggtgggcat gggtcagaag gattcatatg tgggcgatga
 181 ggcccagagc aagagaggca tcctcaccct gaagtacccc atcgagcacg gcatcgtcac
 241 caactgggac gacatggaga agatctggca ccacaccttc tacaatgagc tgcgtgtggc
 301 tcctgaggag caccccgtgc tgctgaccga ggccccctg aaccccaagg ccaaccgcga
 361 gaagatgacc cagatcatgt ttgagacctt caacacccca gccatgtatg tggccatcca
 421 ggctgtgctg tccctgtacg cctctggccg taccactggc atcgtgatgg actccggtga
 481 cggggtcacc cacactgtgc ccatctacga ggggtatgcc ctcccccatg ccatcctgcg
 541 tctggacctg gctggccggg acctgactga ctacctcatg aagatcctca ctgagcgcgg
 601 ctacagcttc accaccacgg ccgagcggga aatcgtgcgt gacattaagg agaagctgtg
 661 ctatgtcgcc ctggacttcg agcaggagat ggccacggcg gcttctagct cctccctgga
 721 gaagagctac gagctgcccg atggccaggt catcaccatt ggcaatgagc ggttccgctg
 781 ccctgaggct ctcttccaac cttccttcct gggcatggag tcctgtggca tccatgaaac
 841 taccttcaac tccatcatga agtgtgacgt ggacatccgt aaagacctgt acgccaacac
 901 agtgctgtct ggtggcacca ccatgtaccc tggcattgct gacaggatgc agaaggagat
 961 tactgccctg gcgcccagca cgatgaaaat caagatcatt gctcctcctg agcgcaagta
1021 ctctgtgtgg atcggcggct ccatcctggc ctcgctgtcc accttccagc agatgtggat
1081 cagcaagcag gagtatgacg agtctggccc ctccatcgtc caccgcaaat gcttctag
```

PRIMER SET #1: 103 bp

PRIMER SET #3: 409 bp

Figure 2.8 **Actin 1 and Actin 3 primer sets design.** The Actin 1 primer set (103 bp amplicon) and the Actin 3 primer set (409 bp amplicon), were designed using the mRNA sequence for beta-actin in *Cercopithecus aethiops*.

generated off Vero DNA for those primer sets (Figure 2.3). Initially, we created three internal control oligonucleotides, "Alu 1" (132 bp amplicon), "Actin 1" (103 bp amplicon), and "Actin 3" (409 bp amplicon).

Standard curves were established to optimize the primer concentration, probe concentration, input internal control DNA concentration, and annealing temperature using SYBR Green and then TaqMan (Table 2.9). The internal control standards were set up so that the threshold cycles would line up with the standard curve generated from the same primer sets when using Vero DNA. This amount was calculated by finding the moles of Vero DNA in a 100 nanogram sample, and then using that amount to calculate the nanograms of each internal control needed to have the same molar amount. The R^2 and efficiency of each reaction are shown in the tables below, which are ways to determine whether or not the assay is fully optimized. The R^2 value demonstrates the linearity of the standard curve points, and the accuracy of the triplicates in the standard curve. An R^2 value of 1 means that all the standard curve point's lie on the line. When a real time qPCR assay is optimized, the R^2 value should be between 0.98 and 1 The amplification efficiency is another way to determine whether or not a real time qPCR assay is optimized. The efficiency is calculated from the slope of the standard curve. Efficiency is equal to $10^{-1/slope}$ and is usually represented as a percentage where percent efficiency is equal to (E-1) x 100%. An optimized reaction will have an efficiency of 90-105%. When the efficiency is less than 90%, it could indicate the reaction conditions were not optimized yet, or the primers were not designed well. If the efficiency is greater than 105% it could be a sign of pipetting errors or co-amplification of a nonspecific product.

(A) Alu 1: 132 bp

I.C. standard	CT
4.85E-1 ng	7.21
4.85E-2 ng	12.12
4.85E-3 ng	15.78
4.85E-4 ng	19.37
4.85E-5 ng	23.28
4.85E-6 ng	27.16

R^2= 0.998
Efficiency: 80.1%

(B) Actin 1: 103 bp

I.C. standard	CT
3.85E-5 ng	20.57
3.85E-6 ng	23.92
3.85E-7 ng	27.45
3.85E-8 ng	31.27
3.85E-9 ng	35.71
3.85E-10 ng	36.84

R^2= 0.985
Efficiency: 92.9%

(C) Actin 3: 409 bp

I.C. standard	CT
8.69E-6 ng	24.46
8.69E-7 ng	27.18
8.69E-8 ng	31
8.69E-9 ng	34.49
8.69E-10 ng	35.68
8.69E-11 ng	37.02

R^2= 0.946
Efficiency: 140.8%

Table 2.9 **Internal control DNA standard curves for Alu 1, Actin 1, and Actin 3 primer sets using TaqMan.** The standard curve with C_T values, R^2, and efficiency. (A) The Alu 1 internal control. (B) The Actin 1 internal control. (C) The Actin 3 internal control.

The Alu 1 and Actin 1 primer sets were optimized at this point. All points on the standard curve could be detected in the linear range. The Actin 3 primer set was also optimized but was not as sensitive as the other primer sets, as it could not detect the lower amounts of the standard curve.

2.4D. ALU 1 PRIMER SET TESTING: SYBR GREEN AND TAQMAN

The Vero cell DNA standard curve was tested in three separate experiments to determine the reproducibility of the Alu 1 primer set in SYBR Green based qPCR (Table 2.10). The data generated by the standard curves over three separate days were very reproducible. As mentioned previously, the Alu primer sets were shown to amplify even the smallest amount of contamination in the no template control (NTC) samples. In the "no template control" (NTC) samples that contained only enzyme master mix, primers, and water, small amounts of DNA were amplified of the same size as the expected amplicon. Even when different primer sets were used, the predicted amplicon size was generated in the NTC, leading us to conclude that the Alu primer sets are so sensitive that they can detect small amounts of contaminating primate/human DNA. The amount of contaminating DNA that was routinely detected in the Alu 1 primer set experiments was about 5×10^{-6} ng. This value was calculated by using the equation generated from the standard curve and the C_T value. Two agarose gels were run of the Alu 1 product NTC samples (Figure 2.11). In the first gel (A), samples from the Vero DNA standard curve were run alongside the NTC samples. The two samples are similar in size. The sample in the second gel (B), in lane 4 is a NTC sample, but with the Alu 1 forward primer and the Alu 3 reverse primer. The expected amplicon size when amplifying Vero DNA is about 160

Standard	CT	CT	CT
100 ng	4.79	4.87	4.88
10 ng	8.71	8.64	8.53
1 ng	11.65	12.21	12.21
0.1 ng	15.25	15.97	15.98
0.01 ng	18.79	19.72	19.6
0.001 ng	22.33	23.59	23.36

Table 2.10 **Alu 1 primer set using SYBR Green to test reproducibility:** The reproducibility of the Alu 1 primer set was tested on three separate days. The C_T values are directly proportional to the amount of DNA present in the sample. Each standard curve was generated by fresh dilutions from the concentrated Vero DNA stock solution.

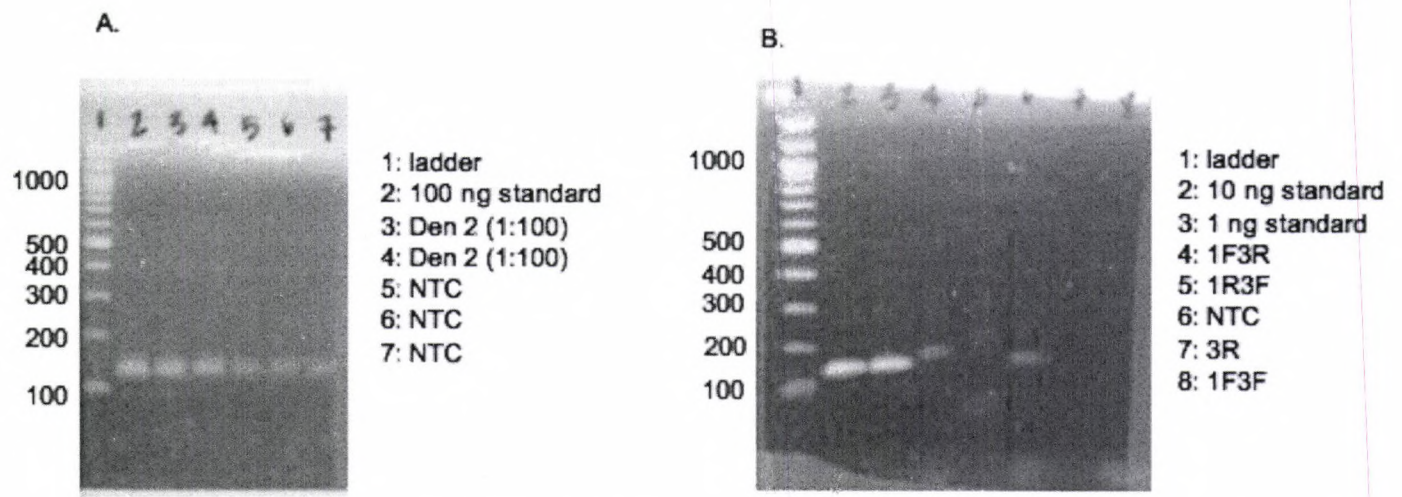

Figure 2.11 **Alu 1 Primer set no template control contamination:** The Alu 1 primer set consistently detects DNA in the no template control (NTC) samples. (A) Alu 1 primers amplifying standard Vero DNA (lane 2), vaccine virus samples (lanes 3 &4), and the NTC (lanes 5-7). (B) Combinations of Alu primer sets. Standard Vero DNA (lanes 2 & 3),NTC (lane 6). Different combinations of Alu primer sets were tested to analyze amplification sizes. Lane 4; Alu1 Forward, Alu3 Reverse , Lane 5; Alu 1Reverse, Alu 3Forward, Lane 7 Alu 3Reverse, Lane 8; Alu 1Forward, Alu 3Forward.

basepairs, which looks to be the size of the amplicon generated in the NTC sample. This would also indicate that the Alu primer sets are amplifying some sort of contaminating human/primate DNA. The amount of Vero DNA in this sample, with primers Alu1 forward and Alu3 reverse as calculated based on the standard curve equation, is about 7 x 10^{-9} ng of DNA, an extremely low amount. The amount of contaminating DNA is extremely low. The Alu 1 primer set was tested using the Taqman protocol and the FAM probe designed for this experiment (Figure 2.3 probe design). A standard curve was generated as done previously for the SYBR Green reactions (Table 2.12). Although the Alu 1 primer set is not as sensitive when using the Taqman protocol as SYBR Green (100 ng standard has a C_T of about 4.8 when using SYBR Green and 10.6 when using Taqman), it is still sensitive enough to detect the 0.01 ng, which is the limit of detection.

2.4E. Actin 1 Primer Set Testing: SYBR Green and Taqman

An experiment was performed to test the reproducibility of the Actin 1 primer set (Table 2.13). This was done for the same reason as the Alu 1 primer set repeats, to test reproducibility of the standard curve when done on three separate days. The results are about the same, showing reasonably consistent C_T values when done over multiple days.

The Actin 1 primer set was also tested using the Taqman protocol with a different FAM probe designed specifically for the actin sequence in Vero DNA (Table 2.14). The standard curve 0.001 ng sample was not detectable, but the 0.01 ng was detected, which is the cutoff for detection. As with the Alu 1 primer set, the C_T at which the 100 ng

Vero Std	CT
100 ng	10.66
10 ng	14.07
1 ng	15.85
0.1 ng	20.91
0.01 ng	25.12
0.001 ng	28.12

R^2= 0.999
Efficiency:

Table 2.12 **Vero DNA Standard curve amplified by Alu 1 TaqMan primer/probes.**

Standard	CT	CT	CT
100 ng	18.93	19.55	19.45
10 ng	22.07	22.29	22.23
1 ng	25.54	25.88	25.9
0.1 ng	29.08	29.53	29.69
0.01 ng	32.42	33.13	34.36
0.001 ng	38.01	36.31	N/A

Table 2.13 **Actin 1 primer set using SYBR Green to test reproducibility:** The reproducibility of the Actin 1 primer set was tested on three separate days. The C_T values are directly proportional to the amount of DNA present in the sample. The standard curves were run with new dilutions from the 100 ng/ul stock solution each time. The Actin 1 primer set shows consistency over multiple days when using the SYBR Green method for product quantification and detection.

Vero Std	CT
100 ng	23.21
10 ng	26.47
1 ng	29.26
0.1 ng	35.89
0.01 ng	37.94
0.001 ng	N/A

R^2= 0.994
Efficiencv: 89.4%

Table 2.14 **Vero DNA Standard Curve amplified by Actin 1 TaqMan primer/probes.**

standard was detected is later (larger number) with the Taqman protocol than with the SYBR Green protocol (23 vs. 19, respectively).

The percent recovered after extraction over the Qiagen column was calculated from the Actin 1 Taqman experiment (Table 2.15). A known amount of internal control DNA (3.85×10^{-6} ng) was added to each of the virus samples before extraction. The output DNA was calculated based on the standard curve generated using the Taqman protocol and internal control probes. The percent recovery varied from 49-116%. These are preliminary data that would need to be repeated several times to generate an average for the 109 bp amplicon percent recovery when extracting over a column.

2.4F. Actin 3 Primer Set Testing: SYBR Green and TaQman

The sensitivity of the Actin 3 primer set was less than the Alu primer sets. It has been shown that increasing the amount of magnesium in the master mix (from 2.5 mM to 3.5 mM) can increase sensitivity and reaction efficiency. The Actin 3 primer set, which generates a 409 bp amplicon, was tested using the SYBR Green method with and without extra magnesium added to the master mix. A standard curve generated using the original amount of magnesium in the master mix ($-Mg^{+2}$) is next to a standard curve generated when using additional magnesium in the master mix ($+Mg^{+2}$) (Table 2.16). The sensitivity of the reaction increased when the additional magnesium was added to the master mix. The detection limit that we need to reach (0.01 ng) is detected at C_T 32.21 when no additional magnesium is added, whereas it is detected at C_T 30.82 when the

Actin 1-IC		
Input DNA	Output DNA	% Recovery
3.85E-6 ng	4.47E-06	116%
	1.90E-06	49.35%
	2.39E-06	62.10%
	2.69E-06	69.87%

Table 2.15 **Recovery of Actin 1 internal control samples.** The percent recovery of the Actin 1 internal controls was calculated after purification from Qiagen DNA column. A known Internal Control (IC) input oligonucleutode was applied to the column, and the amount recovered in the eluate was analyzed using Taqman qPCR. Output DNA was calculated from the Vero Standard curve generated during the qPCR reaction.

A + Mg

Vero Std	CT
100 ng	16.62
10 ng	20.09
1 ng	23.51
0.1 ng	27.97
0.01 ng	30.82
0.001 ng	30.94

R^2= 0.944
Efficiency: 110.5%

B -Mg

Vero Std	CT
100 ng	16.49
10 ng	19.13
1 ng	22.85
0.1 ng	26.96
0.01 ng	32.21
0.001 ng	N/A

R^2= 0.984
Efficiency: 85.5%

Table 2.16 **Effects of additional Mg in PCR reaction using SYBR Green.** (A) Standard curve using Actin 3 primer set when additional magnesium is added to the master mix (3.5mM Mg per reaction). (B) Standard curve using Actin 3 primer set when no additional magnesium is added to the master mix (2.5mM Mg per reaction).

magnesium is added. The efficiency of the experiment is also increased when extra magnesium is added.

The Actin 3 primer set was also tested when using Taqman method for real time qPCR (Table 2.17). This experiment was not able to detect the 0.01 ng in the standard curve, which is the limit of detection for the WHO and FDA. The efficiency and R^2 values are not what would be seen if the parameters were optimized, so further optimization of the Actin 3 Taqman protocol would need to be done in order to detect the 0.01 ng standard sample. More magnesium could be added to the reaction mix, as well as slightly adjusting the Taq DNA polymerase and/or primer concentrations.

2.4G. GROWTH CURVE SAMPLE TESTING

The next step was to test actual vaccine virus samples for the amount and size of residual Vero cell DNA. The real time qPCR assay needs to be able to test for the total amount of Vero DNA in the samples, as well as the size of the residual DNA (Figure 2.18). The sample used to test the assay was DENVax2-P2 (Passage 2) from a previously performed growth curve. A standard growth curve consists of allowing the dengue vaccine virus to infect and grow in a monolayer of Vero cells over a period of 12-14 days without changing the medium. Growth curve samples were tested on day 5 and day 11. During DENVax manufacture, the medium would be changed and cell monolayer washed multiple times, reducing the amount of residual Vero DNA in the samples at later growth days. Since the medium was not changed during this growth curve, it would be expected that day 11 would have more Vero DNA in the sample compared to day 5. The

Vero DNA Standard	CT
100 ng	29.77
10 ng	31.37
1 ng	32.4
0.1 ng	37.13
0.01 ng	N/A
0.001 ng	N/A

R^2= 0.836
Efficiency: 156.5%

Table 2.17 **Actin 3 primer set testing using Taqman Method.** The standard curve of the Vero DNA detection when using Actin 3 primer set, and when additional magnesium is added to the master mix (2.5mM -> 3.5mM).

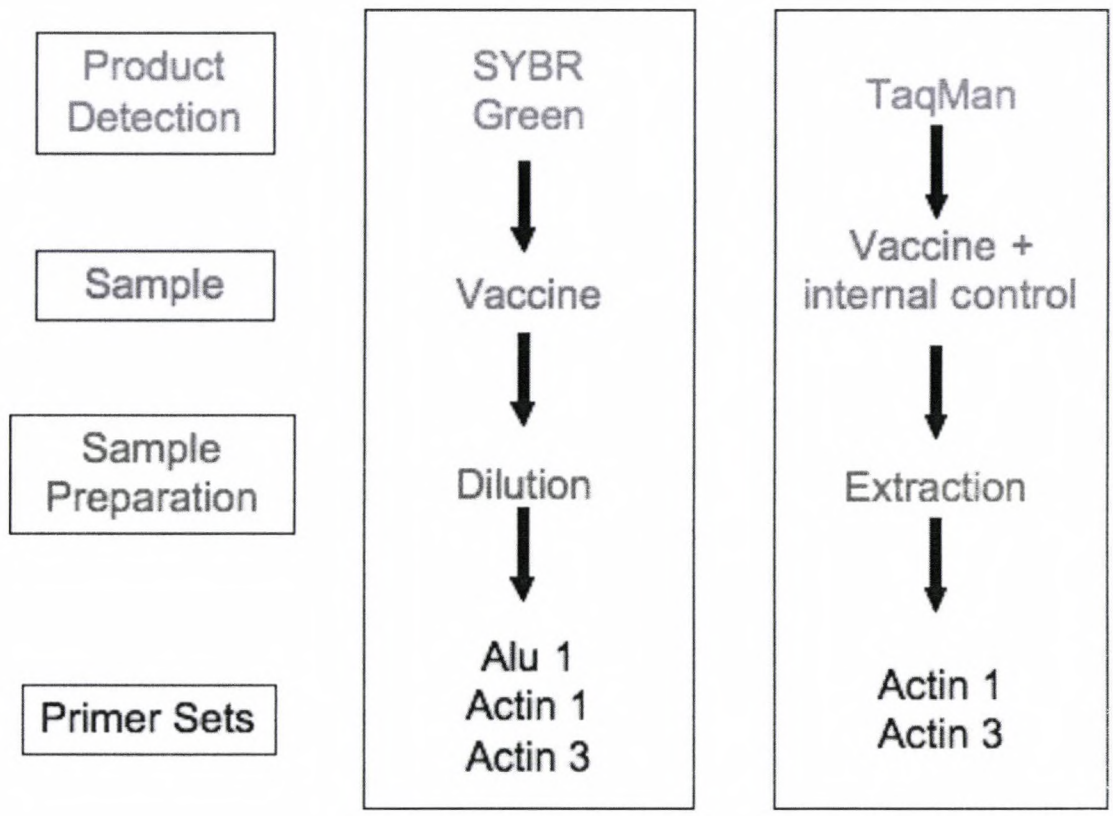

Figure 2.18 **Diagram of questions being addressed by real time qPCR assay.** When using the SYBR Green method for qPCR product detection, the samples are diluted 1:1000 and quantified using Alu 1, Actin 1, and Actin 3 primer sets. When using the Taqman method for qPCR product detection, the internal control samples are added to the sample before extraction and then quantified with the appropriate primer/probe set, Actin 1 or Actin 3.

experiments with each of the primer sets (Alu 1, Actin 1, and Actin 3) were performed on the same day, with the same samples, so the effects of multiple freeze/thaw cycles would not alter the results (Table 2.19).

The samples were RNase treated and then a small aliquot was serially diluted 1:1000 in water. The same dilution samples were used in SYBR Green reactions for each of the three primer sets. As expected, the virus samples had much more Vero DNA on day 11 than on day 5. Also as expected, the amount of Vero DNA is much less when quantifying a 409 bp fragment (Actin 3) than when quantifying ~100 bp fragments (Alu 1 and Actin 1). This demonstrates that the Vero cell DNA that is found in the virus samples has a higher concentration of smaller fragments, than larger fragments. The ratio of Actin 1: Actin 3 in the samples was determined (Table 2.19). The ratio demonstrates that there is a larger amount of smaller amplicons (Actin 1) to larger amplicons (Actin 3) at the later harvest day (day 11).

To test the Taqman protocol, a known amount of the appropriate internal control (Actin 1-IC or Actin 3-IC) was added to the virus sample before extraction with the Qiagen column. The Vero DNA standards worked well for primer sets, but the internal control standards did not work.

2.5. Discussion

The WHO and FDA are concerned about both the size and the amount of residual host cell DNA that is present in virus vaccines. A quantitative real time PCR method has been developed that can address both of these issues. The preparation of the DNA from the

Vaccine sample	ng Vero DNA/ul			Ratio of Actin 1: Actin 3
	Alu 1 (132 bp)	Actin 1 (103 bp)	Actin 3 (409 bp)	
Early Harvest a	6.44	7.85	2.05	3.93
Early Harvest b	7	9.35	2	4.68
Late Harvest a	19	26.6	4.05	6.57
Late Harvest b	20	27.9	4.84	5.76

Table 2.19 **Residual Vero DNA quantification in research-grade DENVax virus samples analyzed with SYBR Green qPCR.** Research grade DENVax-2 virus samples were tested comparing the three primer sets Alu 1, Actin 1, and Actin 3. Duplicate flasks of the infected cells (a, b) were analyzed for their DNA content from days 5 (early harvest) and 11 (late harvest) with separate harvests on each day. An increase in Vero DNA levels from day 5 to day 11 is expected as the growth medium was not changed from days 5-11.

vaccine sample proved to be a key step in accurately estimating the size and amount of Vero DNA. We found that both the Vero DNA standard and virus vaccine samples were contaminated with RNA. RNase treatment effectively removed the contaminating RNA, thus enabling accurate DNA concentration determination. It was also determined that the vaccine storage medium interferes with the qPCR reaction. To obtain an accurate detection of DNA the samples needed to be either diluted 1:1000 in water, or the DNA must be purified using a Qiagen column. There are advantages and disadvantages to both sample preparation methods. When diluting, the detection method must be sensitive enough to detect low amounts of DNA, down to 0.01 ng per sample. When extracting the DNA using a Qiagen column, there must be an internal control present to calculate a percent recovery to apply to the samples. When performing PCR using the SYBR Green method, it was determined that diluting the samples 1:1000 was still sensitive enough for each of the primer sets, Alu 1, Actin 1 and Actin 3.

The SYBR Green assay of DNA quantification by PCR was proven to be reproducible and optimized based on the efficiency and R^2 data. When testing samples, the SYBR Green method was able to show a decrease in the concentration of larger DNA fragments when using Actin 3 primer set, as compared to the smaller amplicon primer sets Alu 1 and Actin 1 (Table 2.19). In the absence of medium change we would predict an increase in cellular debris as we progress from days 5 to 12 in the duration of growth. This assay also successfully showed an increase in DNA amounts on day 11 relative to day 5, since the medium was not changed throughout the virus growth period.

The Taqman method of DNA quantification was able to detect the Vero DNA levels in the virus samples. However, the Vero DNA standard curves were not able to reach the limit of detection that is needed for the FDA and WHO (0.01 ng per qPCR sample). Additionally, when using both probes (duplexing) in the reaction mix, the internal control standards and Vero DNA standards were not linear and the samples were not consistently detected. The internal control samples are needed for the assay to work, because the percent recovery off of the Qiagen column needs to be calculated to apply to the virus samples.

Future studies for this project include optimizing the duplexing reaction in the Taqman protocols for the Actin 1 and Actin 3 internal controls and standard Vero DNA. Upon addition of both probes the efficiency and C_T values do not correspond to values seen when only one probe is used in the reaction. This indicates there is some interference between the probes and template DNA that is not allowing accurate detection of our known input DNA. This interference is seen in both the standard Vero DNA as well as the synthetic DNA internal controls. It is possible that we may need to change the reaction conditions including Mg^{+2}, dNTP, and enzyme. Ideally, upon optimization of this assay we could imagine greater sensitivity, as we would concentrate the vaccine sample upon purification off the Qiagen purification column. However, without understanding the recovery trends and percent loss off the column, it is hard to be confident in the results we are observing.

With this assay optimized, we can implement DNA quantification in the manufacturing of future DENVax vaccine lots. Validation of this assay could occur by performing

multiple replicates by multiple users. Many vendors offer a GLP (Good laboratory practice) certified test for residual host cell DNA. Eventually we would compare the results from our assay described here to other assays available commercially.

CHAPTER 3

DESIGN & CHARACTERIZE A SECOND-GENERATION CHIMERIC DENVAX4 VACCINE

3.1. BACKGROUND AND RATIONALE FOR MAKING SECOND-GENERATION DENVAX4

An effective dengue vaccine must provide complete protection against all four of the dengue serotypes. The current DENVax4 vaccine has lower replication efficiency and is not as immunogenic as the other vaccine virus serotypes (manuscript in preparation). The immune response generated in mice and non-human primates to DENVax4 in the tetravalent formulation is limited as compared to the other, more immunogenic vaccine viruses (DENVax-1, -2, -3). The goal of re-engineering the infectious cDNA clones of DENVax4 is to boost replication efficiency *in vitro* and *in vivo*, and boost immunogenicity in the host. Previous research conducted by Claire Huang and Rich Kinney at the CDC on the chimeric DEN-2/West Nile vaccine showed that replication could be improved by modifying the amino acid sequence upstream of the C/prM cleavage site (*40*). The hypothesis is that by changing the amino acid sequence upstream of the C/prM cleavage site to be more like the wild type dengue-4 serotype as opposed to the dengue-2 like the current DENVax4, it will increase replication *in vivo* and *in vitro*.

Although replication *in vitro* is not necessarily directly linked to replication efficiency, it is hypothesized to be the reason for the less robust anti-DENVax4 immune response.

Dengue viruses have an RNA genome and it is known that the secondary structure of RNA plays an enormous role in its function and stability. When RNA is translated into protein, the secondary structure also affects protein expression levels (*41*). Three DENVax4 variants were designed with different mutations upstream of the C/prM junction to make the sequence more "dengue-4 like". Since the current chimeric DENVax4 has the native dengue-2 sequence 5' of the C/prM junction and dengue-4 sequence downstream in the prM/E gene region, the secondary structure formed by the RNA is not identical to wild type dengue-4 virus. By making both regions "dengue-4 like", the secondary structure formed from the RNA will be more similar to the wild type dengue-4 virus, which will hopefully result in improved translation and intracellular viral maturation. The change in amino acid sequence could also be hindering the cellular protease/signal peptidase from efficiently cleaving the viral polyprotein at the C/prM junction. The replication efficiency *in vivo* and *in vitro* is likely to increase due to the improvement in polyprotein cleavage and viral maturation.

We generated variants termed DENVax4-b, c, and d. Figure 3.1 shows the amino acids changed in each DENVax4 variant. The DENVax4-b variant contained 6 amino acid changes 5' of the C/prM junction and one amino acid change 3' of the junction. The DENVax4-c variant contains those same mutations as well as two other amino acid changes 5' of the C/prM junction that make it even more like the DENV-4 wild type virus. The DENVax4-d variant has an amino acid deletion 5' of the C/prM junction,

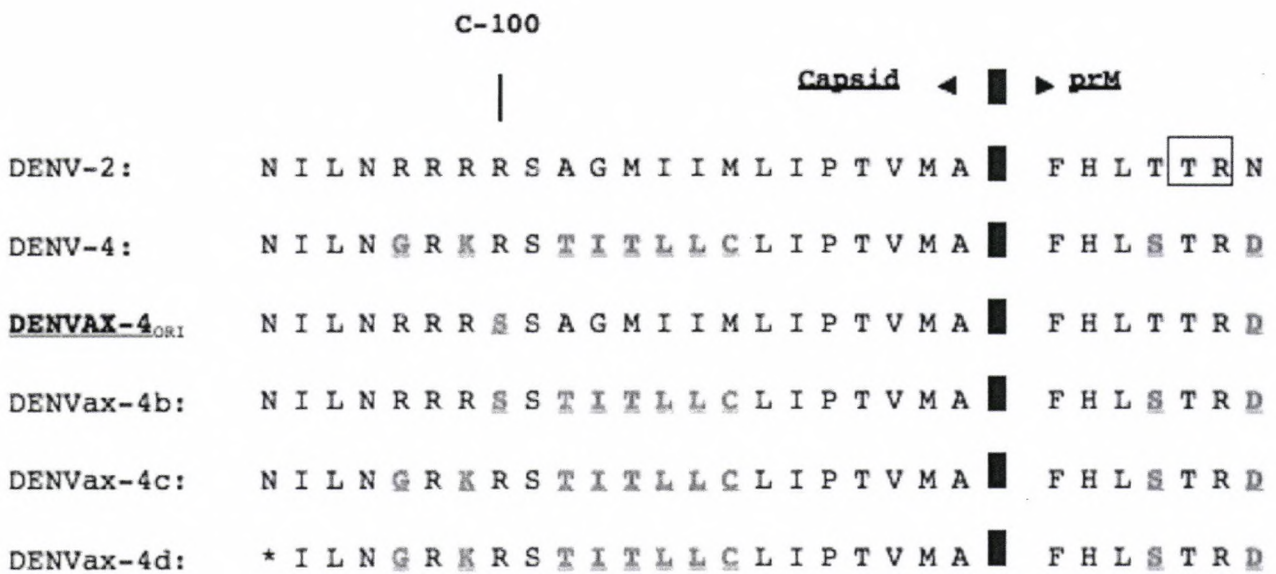

Figure 3.1 **Amino acids changes in the DENVax4 variants.** The capsid/prM junction amino acid sequences for the original DENVax4 and the second generation variants, DENVax-4b, DENVax-4c, and DENVax-4d. The black box represents the capsid/prM cleavage site. **DENVax-4*ori*** is the original DENVax-4 vaccine virus. The underlined and bolded amino acids were modified from DENVax-4_{ori}.The boxed TR is the MluI restriction enzyme site.

which causes a shift in the sequence. The shift causes position C-100 in the capsid protein to be a serine, which has already been shown to enable successful replication in mammalian cells (*24*).

3.2. Cloning Overview

Dr. Claire Huang at the CDC generously provided the original cDNA infectious clones. The mutations made in the second-generation DENVax4 variants were between two unique restriction sites in the infectious clone, AgeI and MluI (Figure 3.2). A synthetic fragment was designed for each of the three variants between the AgeI and MluI restriction sites (about 484 bp), and synthesized by GenScript (Piscataway, NJ). The cloning strategy involved multiple intermediates because the entire infectious clone was almost 13Kb, and *E. coli* containing larger plasmids is poorly viable. The cloning overview to generate DENVax4-b, c, and d infectious clones is shown in Figure 3.3. The first step in the cloning process involved inserting the AgeI to MluI synthetic fragment into a subclone pD2/3-PP1-5. The product is termed pD2/3/4i-Age/Mlu. The next step was to insert the MluI to NgoMIV fragment from the current pDENVax4 infectious clone into the newly made pD2/3/4i-Age/Mlu, which we named pD24i. The last step was to move the NgoMIV to XbaI fragment from the current pDENVax4 full clone into the pD24i. This resulted in three separate full-length infectious clones.

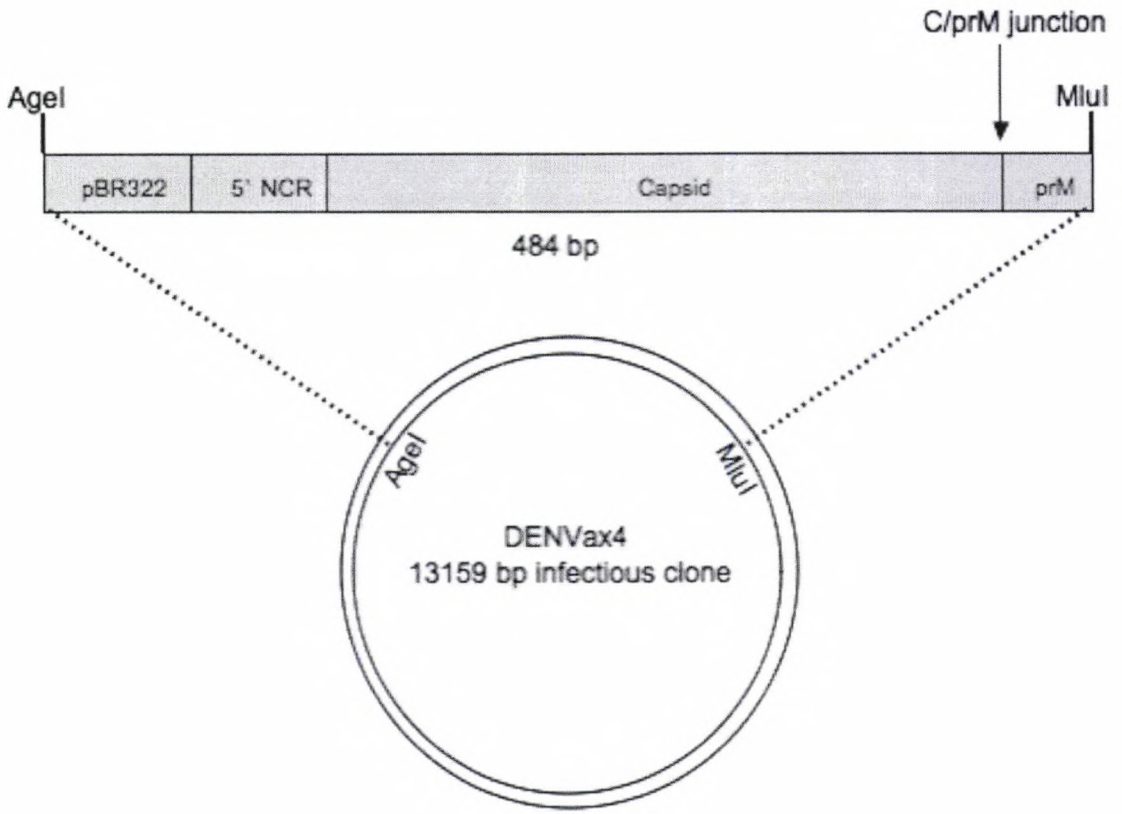

Figure 3.2 **Plasmid map of DENVax4 2nd generation infectious clones.** The AgeI to MluI synthetic fragment includes part of pBR322 cloning plasmid, 5' NCR, capsid and prM gene segments of each variant.

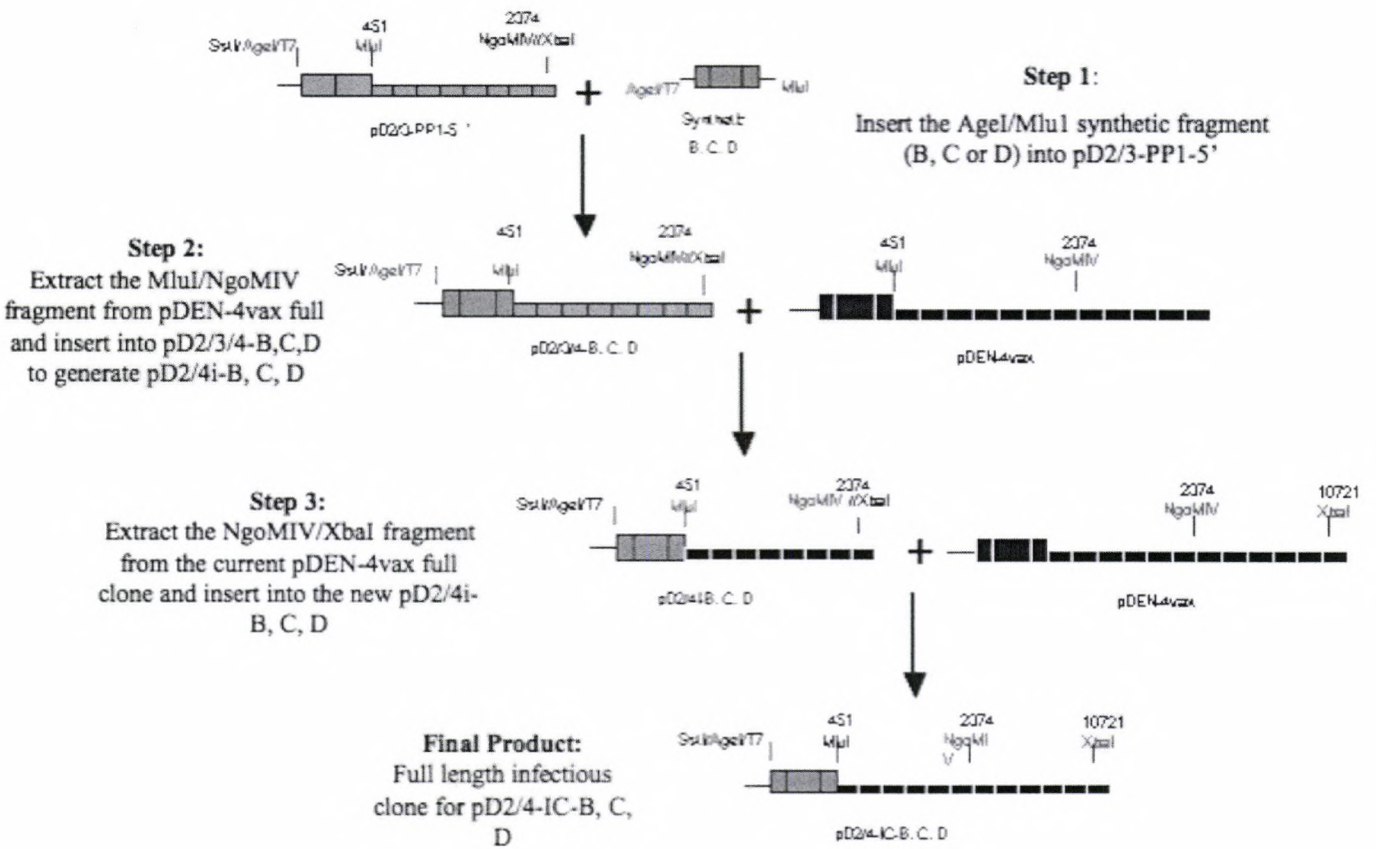

Figure 3.3 **Cloning overview to generate DENVax4-b, c, d infectious clones.** Steps 1-3 in the cloning procedure to generate the full length infectious clones for each variant. The synthetic fragments made by Genscript were the AgeI to MluI segment that contained the mutations specific to each DENVax-4 variant.

3.3. Materials and Methods

3.3a. Synthetic fragment synthesis

GenScript (Piscataway, NJ) generated the synthetic inserts flanked by AgeI and MluI restriction sites. These inserts contained the mutations 5' of the C/prM junction specific to each variant. The synthetic fragments were provided in a pUC57 backbone.

3.3b. Ligation/Transformation

The ligation/transformation protocol for the second-generation DENVax4 used XL1 Blue *E. coli* competent cells (Strategene, La Jolla, CA). In previous studies, the XL1 Blue cell line was found to improve the stability of large plasmids. The competent cells were placed on ice to thaw from -80°C for 15 minutes. While the cells were thawing the ligation reaction was assembled containing T4 DNA Ligase, DNA Ligase Buffer, template DNA, and water to a total reaction volume of 20 µl. A "no insert" control for each sample was included to test if the vector would re-ligate with itself without an insert present. The ligation reaction was incubated at room temperature (20-25°C) for ten minutes. The competent cells were split into 50 µl aliquots per reaction, and 2 µl of the ligation reaction was added to the 50µl of cells and the mixture was tapped gently to mix. The cells and ligation reaction were incubated for 30 minutes on ice, followed by incubation at 42°C for 20 seconds, and then placed on ice for an additional 2 minutes. 2XYT medium was added and then incubated on a shaker at 37°C for one hour. The

samples were then plated on 2XYT plates + ampicilin and incubated at 37°C overnight. The next day colonies were picked and grown up in 2XYT media with ampicilin.

3.3C. *IN VITRO* TRANSCRIPTION- AMPLISCRIBE

The plasmids containing the full-length infectious cDNA clones were digested with the restriction enzyme XbaI, to linearize the plasmid in preparation for transcription. The digestion was performed at 37°C and DNA was purified using the GENECLEAN® *Turbo* purification kit (MP Biomedicals, Solon, OH). From previous experience we found that the concentration of template DNA and the concentration of ATP in the reaction mixture were very important (personal communication, Dr. Huang). If the template DNA concentration is too high and the ATP concentration too low, unwanted mutations will be added into the RNA due to the lack of sufficient ATP. Therefore, towards the end of the transcription reaction, nucleotides other than "A" are incorporated. The reaction conditions were optimized to use 200 nanograms of template DNA and 1/5 the recommended ATP concentration as the other NTPs to promote efficient capping of the mRNA as well as correct addition of adenosine into the RNA sequence. We used the AmpliScribe™ T7 High Yield Transcription Kit (EPICENTRE Biotechnologies, Madison, WI) to make RNA from DNA cloned downstream from a T7 RNA polymerase promoter. The reaction mixture contained 200 nanograms of linearized template DNA, Capping NTP solution (25mM CTP, 25mM GTP, 25mM UTP, and 5mM ATP), 10X T7 Buffer, A-Cap Analog, 10mM DTT, and Ampliscribe Enzyme). The A-Cap analog, $m^7G(5')ppp(5')G$ RNA Cap Structure Analog, was not provided with the kit so it was

ordered separately (NewEngland Biolabs, Ipswich, MA). The cap structure increases stability of the mRNA by decreasing susceptibility to degradation by exonucleases.

3.3D. ELECTROPORATION

Electroporation of the DENVax4-b, c, and d RNA was performed at the Centers for Disease Control and Prevention (CDC) in Dr. Claire Huang's laboratory. The RNA transcription and Vero cell preparation were performed on the same day as Vero cell electroporation. Confluent flasks of Vero cells were trypsinized, washed, and resuspended in RNase free/sterile PBS at a concentration of $1x10^7$ cells/ml. For the electroporation reaction, 400 µl of cell suspension ($4x10^6$ cells total) was added to the viral RNA in a 1.7 ml eppendorf tube. The cells and RNA mixture were assembled in a pre-chilled metal cuvette and electroporated using a single square wave 25 msec pulse with a voltage of 225V in a 4mm cuvette. After the pulse, the mixture was transferred to a new eppendorf tube using a sterile glass Pasteur pipette and incubated at room temperature (20-25°C) for approximately fifteen minutes. The mixture was transferred to a T-75 cm^2 flask containing pre-warmed medium, DMEM 10% FBS at 37°C. The next morning the medium was replaced with DMEM 5% FBS, 0.1% F127. Cells were monitored for the first few days for viability after electroporation. Viruses were grown for 12 days, and Vero cell cytopathic effects (CPE) were monitored for the duration of growth.

3.3E. CELL CULTURE

Vero cells obtained from Shantha Biotechnics were grown in T-185 cm^2 flasks in DMEM and 10% fetal bovine serum. New Zealand derived FBS was used when culturing the Vero cells and growing the viruses, to minimize the chance of contaminating bovine spongiform encephalopathy (BSE) prions. For passaging, each week the cells were trypsinized using TrypLE^{TM} Express (Invitrogen, Carlsbad, CA) and diluted 1:12 before being added to new T-185 cm^2 flasks. The cells were maintained through 20 passages before new cells were thawed from liquid nitrogen.

The C6/36 cell line used in the growth curve was purchased from ATCC (Manassas, VA), catalog number CRL-1660^{TM}. The C6/36 cells were derived from *Aedes albopictus* mosquito larvae and are adherent in culture. The cells were grown in DMEM with 10% Hyclone FBS at 29°C.

3.3F. GROWTH CURVES

Growth curves were performed to monitor viral growth characteristics and to compare the original DENVax4 to the second-generation candidate DENVax4 viruses. Vero cells were plated in T-75 cm^2 flasks in DMEM with 10% FBS for two day confluency. The cell monolayer was monitored to ensure 100% confluency before virus adsorption. The virus was added at an MOI of 0.001 and incubated for 1.5 hours, rocking every 15 minutes. After adsorption the medium was removed, cells were washed with PBS, and DMEM, 5% FBS, 0.1% F127, 1% pen/strep (1x) was added. Medium aliquots were

taken every other day and then plaque titrated to analyze the growth characteristics of the separate viruses.

3.3G. Plaque Titration of Dengue Virus

Standard plaque titrations with a neutral red overlay were performed to titrate the virus samples. On Day -2, Vero cells were plated in 6-well plates by adding 3 ml of cell suspension at a concentration of $1.7x10^5$ cells/ml. On Day 0 the cell monolayers were checked to ensure 100% confluency before starting the experiment. 10-fold serial dilutions of virus seed in cold BA-1 (1% bovine serum albumin (BCFV), 1X M199 Hank's Salts w/o L-Gln, 0.05M Tris-HCl pH 7.5, 1X L-Glutamine, 0.034% $NaHCO_3$, 1X Pen-Strep, 1X Fungizone (1 mg/ml), MQH_2O to 1L) were performed in a 96-well plate and stored at 4°C until ready for use. The medium was aspirated from each well of a 6 well plate, and immediately inoculated with 100 µl of the appropriate virus dilution. The plates were incubated for 1.5 hours at 37°C with rocking every 10 minutes. After 1.5 hours of incubation, 4 ml of the nutrient/agarose medium (9.8% 10X Earl's balanced salt solution, 3.3% YE-LAH, 2% FBS, 0.225% $NaHCO_3$, 100 mg/2L Gentamycin, 2 mg/2L Fungizone, 0.8% agarose) were added to each well. After incubating at room temperature (20-25°C) for 30 minutes, the plates were incubated at 37°C for 7 days. On Day 7, a 2nd agarose overlay was added to the wells that contained neutral red (3.3 g/L in DPBS), and the plates were incubated at 37°C until plaques were visible, approximately two days.

3.3H. IMMUNOFOCUS ASSAY

An immunofocus assay was performed on the DENVax-4 constructs to titer the viruses after generation of P2. Vero cells were plated in 6-well plates two days prior to the overlay. On Day 0, the plates were infected as described in the standard plaque titration. After the 1.5 hour incubation with virus, the plates were overlayed with 2X nutrient medium and 1.2% Avicel (instead of 0.8% agarose). The plates were incubated at 37°C for 7 days. On Day 7, the cells were fixed with 85% acetone for 30 minutes at -20°C. The plates were put in 1-gallon freezer bags to minimize evaporation of the acetone. The acetone was removed after 30 minutes and transferred to a waste bottle. The plates were washed one time with PBS, and 1 ml of block buffer (wash buffer, 2.5% powdered skim milk, 0.5% Triton X-100) was added to each well. The plates were incubated for 30 minutes at 37°C and then the block buffer was discarded. The primary antibody (monoclonal antibody to the E protein provided by the CDC) was diluted to 1:2000 in block buffer, added to the cells, and incubated at 37°C for 45 minutes. After primary antibody, the plates were rinsed two times with wash buffer, secondary antibody (goat anti-mouse conjugated to alkaline phosphatase) was diluted 1:500 in block buffer, and added to each well for another 45 minutes at 37°C. The plates were rinsed three times with wash buffer before substrate was added until the plaques became visible. The reaction was stopped by gently washing each well with water. The plates were then inverted to dry until counting the plaques.

3.3I. SEQUENCING

Sequencing was performed on the RNA of DENVax4-b and -c P2 viruses. Viral RNA was purified and high quality cDNA generated. Qiagen's viral RNA purification kit was used to extract RNA from the P2 harvest that was stored in 20% FBS at -80°C. The RNA was eluted in 60 µl of Buffer AVE and stored at -80°C until use. RT/PCR using Roche's Titan One Tube RT-PCR System was used for amplification of cDNA from the extracted viral RNA. The master mix contained RNase free H_2O, 5X Buffer, 25mM $MgCl_2$, dNTPs, 100mM DTT, 20µM Forward primer, 20µM Reverse Primer, RNase Inhibitor and enzyme mix. An aliquot of the master mix was added to individual tubes and then the template RNA was added to each sample. The thermocycler program consisted of (50°C/30 min; 96°C/2 min) x 1 reverse transcriptase step, followed by a PCR cycle consisting of (96°C/20 sec; 60°C/30 sec; 68°C/3 min) x 35 and then (72°C/7 min) x 1, and then 4°C hold. There were 5 primer sets to generate a complete overlapping cDNA set, provided by Claire Huang at the CDC (forward and reverse primers: D2/1 & cD2-2670, D2-2625 & cD2-5124, D2-4994 & cD2-7107, D2-7047 & cD2-8890, D2-8798 & cD2-10723). A small aliquot of each cDNA product was checked on an agarose gel to ensure proper amplification. If the cDNA amplification was successful, the DNA was gel purified using the QIAquick Gel Extraction Kit. The concentration of each sample was determined using a nanodrop system and then diluted to the proper concentration for the sequencing reaction. The sequencing reaction used Bigdye, appropriate primer and template DNA diluted to about 30 ng. There were 6-8 sequencing primers for each cDNA amplified. The sequences were analyzed by using the freeware CLC Sequence

Viewer 6 program. The chromatograms were analyzed by using freeware FinchTV software.

3.3J. PLAQUE SIZE

The plaque size is a phenotypic test of attenuation for dengue vaccines. Plaque size was measured by performing a standard plaque titration using neutral red as described previously, and then manually measuring the diameter of the plaques generated on 6-well plates.

3.4. RESULTS

3.4A. GENERATING THE FULL-LENGTH INFECTIOUS CDNA CLONES

The synthetic fragments made by GenScript from AgeI to MluI restriction sites (484 bp) were provided to Inviragen in a pUC57 plasmid. The pUC57 plasmid was digested with AgeI and MluI restriction enzymes so the segment of interest could be separated from the rest of the cloning plasmid. The 484bp fragment was run on a 0.8% agarose gel, was gel purified using a GENECLEAN® Turbo Purification kit, and was eluted in 30 µl of 10mM Tris-HCl. The subclone from step one, pD2/3-PP1-5 also was digested using AgeI and MluI restriction enzymes and the vector was gel purified. A ligation/transformation reaction was performed to insert the new AgeI to MluI synthetic fragment into the pD2/3-

PP1-5 plasmid. The result was one of three plasmids containing gene segments from dengue 2, 3, and 4, therefore they were called pD2/3/4-b, c, and d (Figure 3.4).

The second step involved digesting the pD2/3/4i plasmid and the original DENVax4 with MluI and NgoMIV. The MluI to NgoMIV was excised from the pD2/3/4i plasmid and replaced with the original DENVax4 MluI to NgoMIV section, resulting in a plasmid called pD24i. The third step included digesting the newly made pD24i plasmid and original DENVax4 with NgoMIV and XbaI. The plasmids were gel purified and the pD24i section from NgoMIV to XbaI was removed and replaced with the original DENVax4 NgoMIV to XbaI section. This resulted in the full-length infectious clones for each of the three variants, b, c, and d (Figure 3.4).

Once the full-length infectious cDNA clones were generated, they were sequence confirmed. Unfortunately, we discovered the constructs did not contain the essential 5' noncoding region cytosine to thymidine mutation at nucleotide 57. This 5' NCR-57 nucleotide is one of the three attenuating mutations in the dengue 2 PDK-53 virus backbone (*22*). Therefore the infectious clones needed to contain the thymidine at that position rather than the wild type cytosine.

Instead of regenerating the entire infectious clone, we were able to work with a very small region of our newly generated "incorrect" clone to make the necessary change and restore the attenuating mutation. There were two unique restriction sites flanking the 5' NCR-57 in the synthesized fragments from GenScript in the pUC57 plasmid (Figure 3.5). The pUC57 plasmid was digested with the unique restriction enzymes, BglII and BlpI,

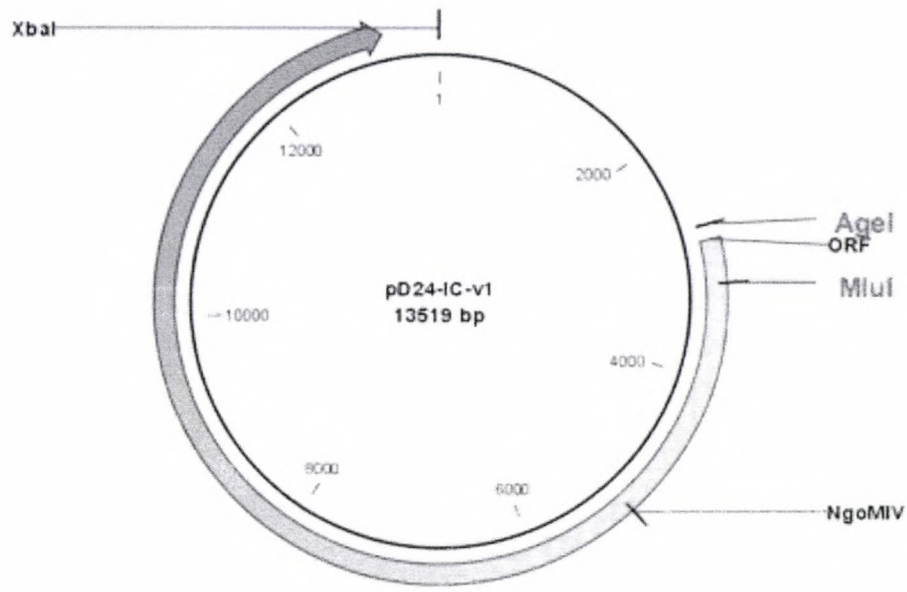

Figure 3.4 **Plasmid map of the full length infectious clones.** The AgeI to MluI segments generated by Genscript for each DENVax-4 variant replaced the respective segments from the DENVax4 full length infectious clone.

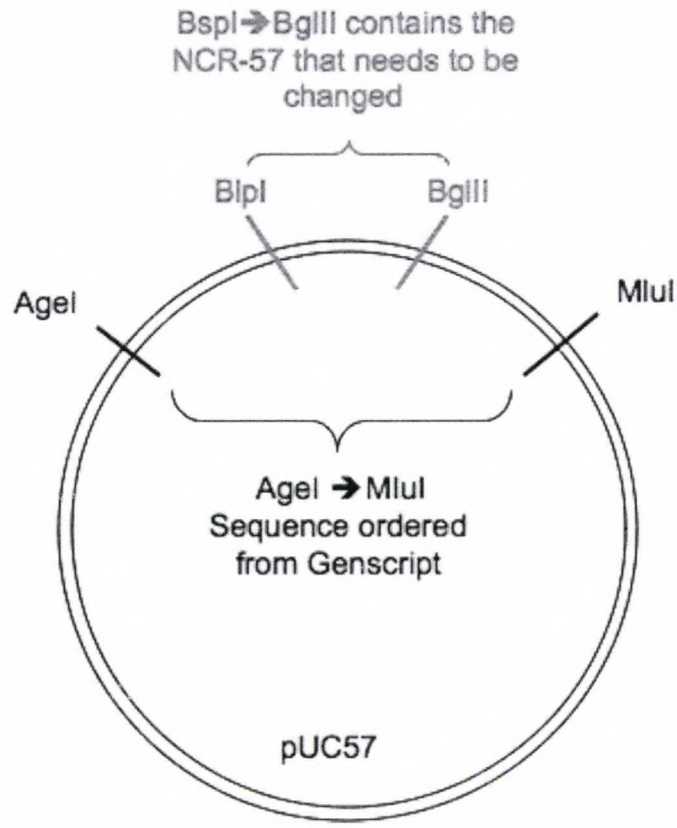

Figure 3.5 **The pUC57 plasmid with unique restriction sites flanking the 5' NCR-57.** The synthetic fragments lacked the necessary 5' NCR-57 attenuating mutation. Two unique restriction sites flank the sequences surrounding the NCR-57 nucleotide, BlpI and BglII. The pUC57 plasmid was digested with these two restriction enzymes and the 48 bp fragment was replaced with a revised oligonucleotide fragment containing the essential attenuating mutation.

which resulted in a 48 base pair fragment. Two complementary oligonucleotides were ordered from Invitrogen that when annealed produced the 48 base pair fragment, to clone into the digested pUC57 vector. These two oligonucleotides had the correct NCR-57 C to T mutation, and were annealed by heating to 95°C and then slowly cooling to room temperature. A transformation and ligation reaction was used to clone the annealed oligonucleotides into the pUC57 vector. The insert segment was sequenced to confirm that the modified fragment containing the attenuating NCR-57 nucleotides was ligated into the vector.

Once the sequences were confirmed, the pUC57 backbone was digested with AgeI and MluI restriction enzymes to isolate the corrected 484 bp fragment. The infectious clones that were previously made were also digested with AgeI and MluI, and the fragment was gel purified. A ligation/transformation reaction was performed to put the correct AgeI to MluI fragment into the infectious clone. The re-generated cDNA infectious clones were sequenced again to confirm the presence of the 5' NCR-57 mutation.

3.4B. VIRUS GENERATION

Digesting with the restriction enzyme XbaI linearized the full-length infectious cDNA clones. The cDNA for each of the three variants, b, c, and d, was transcribed into genomic viral RNA. The RNA was transformed into Vero cells by electroporation, after first using Lipofectamine™ 2000 (Invitrogen) without success (data not shown). The viruses were grown for 12 days while monitoring cytopathic effect (CPE), and harvested. This first harvest after electroporation was termed P1 (Passage 1). The subsequent

amplifications and passages of viruses were called P2, P3, etc. The CPE generated by the P1 viruses was compared to the original DENVax4 virus. There was limited CPE for the original DENVax4, and DENVax-b, and c, whereas DENVax4-d did not generate any noticeable CPE. Unlike other viruses when grown *in vitro*, the dengue viruses do not generate much CPE in Vero cells. Although the DENVax4-d virus did not generate any CPE *in vitro*, we subsequently amplified this in parallel with the other strains.

The P1 viruses (DENVax4-b, c, d P1) were amplified to generate high enough titers to perform sequence analysis and growth curve experiments. To generate P2 (Passage 2), monolayers of Vero cells were blindly infected, without first titering the P1 viruses, with 2 ml of P1 harvest. The P2 viruses were grown for 7 days in Vero cells and harvested on day 5 and day 7. The P2 amplification was titered by an ImmunoFocus assay before starting sequencing analysis or growth curve experiments. DENVax4-b and c reached high titers by day 7 of 1.1×10^6 pfu/ml and 5.5×10^5 pfu/ml, respectively. DENVax4-d had no titer; therefore very low or no virus is capable of replicating in Vero cells. Subsequent experiments were continued with only the DENVax4-b and c viruses.

3.4C. Phenotypic and Genetic Characterization of the Viruses

Phenotypic characterization of the viruses included growth curves in Vero cells and C6/36 (*Aedes albopictus*) cells. Genetic characterization was done by sequence analysis of the DENVax4-b and -c viruses. The growth curve in Vero cells was performed to compare the efficiency and peak titers of the second-generation DENVax4 viruses to the original DENVax4 virus (Figure 3.6). The controls in the experiment were DENVax2-

P2, DENVax4-P2, and DENVax4-P8. The DENVax2-P2 virus was included as a control because it has high replication efficiency, grows to high titers, and was a good standard for comparison in Vero cells as it generates relatively high CPE in Vero cells. The DENVax4-P2 was the GMP manufactured P2 DENVax4 strain. The DENVax4-P2 virus growth curve can be more directly compared to the second-generation viruses because they are also a single passage removed from electroporation. The DENVax4-P8 (passage 8) virus was the surrogate master virus seed and was extensively characterized *in vitro* and *in vivo*. Vero cells were infected at an MOI of 0.001 and the culture was sampled every other day through day 13. Samples from days 3, 5, 7, 9, 11, 13 for each of the viruses were titrated by plaque assay in Vero cells (Figure 3.6). The DENVax2-P2 virus reached peak titers faster than the other viruses, as expected. The DENVax4-b virus was similar in peak titer and growth rate to the DENVax4-P2 and P8 viruses. The DENVax4-c virus was slower growing initially than the other viruses, but reached a similar peak titer.

Growth in C6/36 mosquito cells was evaluated to compare the growth characteristics of the second-generation viruses to the wild type dengue 4 virus (strain 1036) (*42*). To demonstrate attenuation the replication efficiency of the attenuated vaccine viruses should be decreased in C6/36 mosquito cells as compared to the wild type virus (*24*). This phenotype is an essential safety feature of DENVax vaccine viruses, to decrease possible transmission of attenuated chimeric viruses in nature. C6/36 cells were infected at an MOI of 0.001 with each of the P2 viruses (-b, -c, and wild-type) in duplicated flasks and were grown for 14 days. Aliquots of supernatant were sampled every other day starting at day 2, and the samples were titrated by plaque assays in Vero cells. The

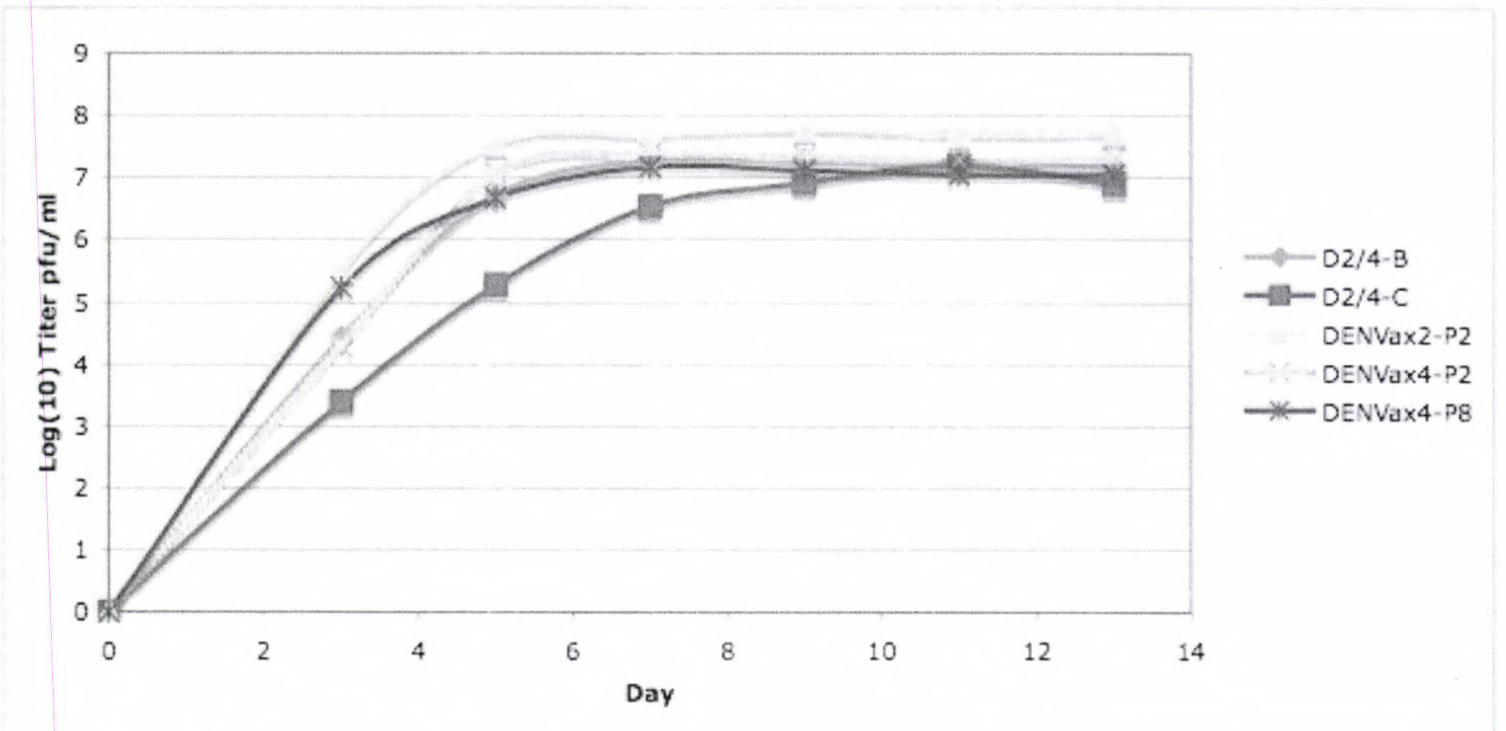

Figure 3.6 **Second Generation DENVax4 Candidate Virus growth in Vero cells.** Growth curves were performed by infecting confluent flasks of Vero cells at an MOI of 0.001 and incubating at 37°C for 14 days. The DENVax4 second generation viruses, DENVax4-b and DENVax4-c were compared to DENVax2-P2, DENVax4-P2, and DENVax4-P8. Samples were taken on days 3, 5, 7, 9, 11, and 13, and titrated by plaque titration.

average titers of the duplicate DENVax4-b and DENVax4-c viruses were compared to the wild type dengue 4 1036 virus (Figure 3.7). The dengue 4 wild type virus (WT D4 1036) replicated most efficiently and to the highest titer, 1.5 x 10^8 pfu/mL by day 14. The DENVax4-b virus replicated reasonably well in the C6/36 cells, reaching a peak titer of 2.7 x 10^6 pfu/mL by day 14, but not as well as the wild type virus. The DENVax4-c virus was very slow growing until after day 6 when growth was accelerated until day 14 and reaching a peak titer of 2.2 x 10^4 pfu/mL. The growth characteristics of the DENVax4-c could indicate an adaptive mutation occurred after day 6 that allowed the virus to grow more efficiently in C6/36 cells. The samples would need to be plaque titrated and then plaques picked on early and late days in the growth curve for sequencing to confirm the hypothesis. At day 6 both of the variants were similarly attenuated for growth compared to the wildtype in C6/36 cells, and were comparable in titer to the original DENVax-4 (*24*).

The plaque size was determined using the virus titration from the Vero cell growth curve. The plaque size of the DENVax4-b viruses was 0.3 cm (an average of 7 plaques) in diameter (Figure 3.8b). The plaque size of the DENVax4-c viruses was 0.1 cm in diameter (an average of 7 plaques) (Figure 3.8c). Pictures of the DENVax4-P2 and DENVax4-P8 viruses were taken as a comparison to the general plaque size and morphology of the second-generation DENVax4 candidate viruses (Figure 3.8a). The plaques in the –b and –c candidate viruses, especially DENVax4-c were not homogeneous. There was a mixed population of viruses with some small and some large plaques.

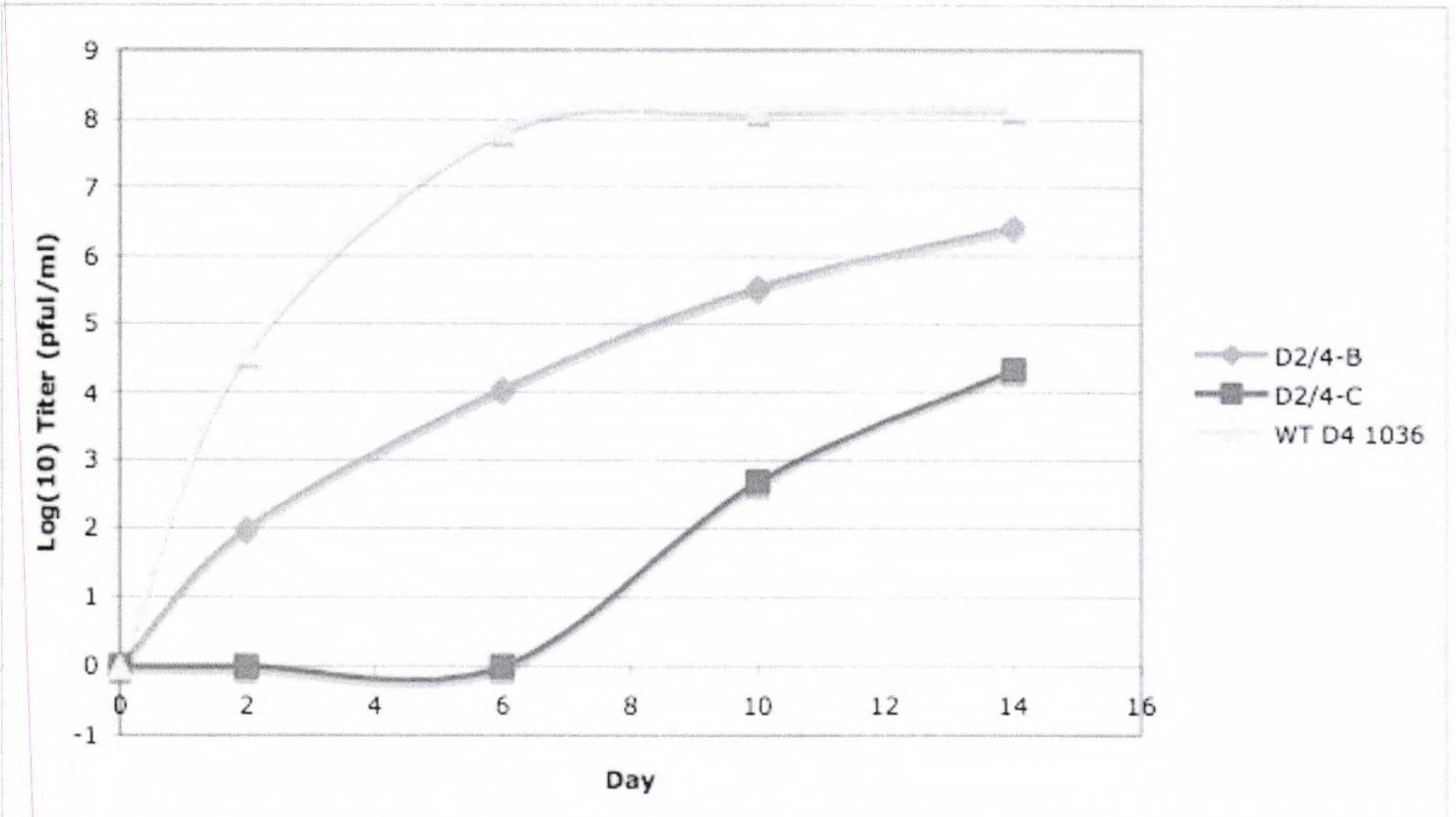

Figure 3.7 **DENVax4 Candidate Virus growth curve in C6/36 cells.** The growth of DENVax4 second generation viruses, DENVax4-b and DENvax4-c were compared to the wild type dengue 4 virus (1036) in C6/36 mosquito cells. Samples were taken on days 2, 6, 10, and 14, and titrated by plaque titration.

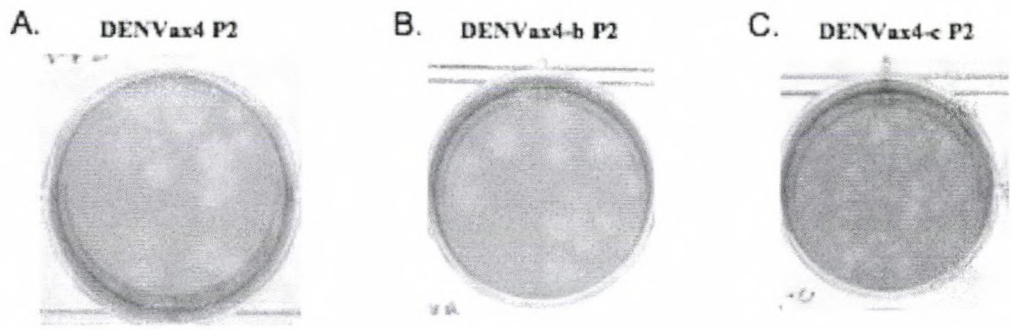

Figure 3.8 **DENVax Plaque Phenotype. P2 seeds of DENVax4 (A) DENVax4-b (B) and DENVax4-c (C) were visualized by standard plaque titrations on Vero cells.** DENVax4-b (B) virus had an average plaque size of 0.3 cm (average of 7 plaques) in diameter. The DENVax4-c (C) viruses had plaque sizes ranging from .05-.15 cm and had an average plaque size of about 0.1 cm (average of 7 plaques) in diameter.

The RNA of the P2 stocks of DENVax4-b and DENVax4-c viruses were fully sequenced and compared to the expected sequence. The mutations found in each virus are summarized in Table 3.9, which shows the expected nucleotides and amino acids, and the mutations.

The DENVax4-b P2 virus RNA had two mutation differences from the expected sequences. Both of the mutations found were mixed virus populations, where both nucleotides could be identified in the chromatograms. Nucleotide 416 is the capsid (near the C/prM junction) of the DENVax4-b virus. Since this was a mixed population, it looked like the nt 416 was mutating into an "A" nucleotide instead of the engineered "G" nucleotide. This caused the expected amino acid cysteine to instead be a tyrosine. The second mutation found in the DENVax4-b P2 virus was at nucleotide 8769. This was also a mixed population, some of the viruses had the expected "A" nucleotide at that position, whereas some contained a "C". This caused a change in the amino acid at the position from the expected glutamine to a proline. This mutation was in the NS5 gene region of the infectious clone.

The DENVax4-c P2 virus had four mutations. They were all complete conversions, unlike the DENVax4-b virus that had mixed populations. The mutation at nucleotide 400 in the capsid region of the genome affected an engineered modification at the C/prM junction. The expected/engineered nucleotide was an "A" but the entire virus population mutated to a "C" at that position. This caused the expected amino acid at that position to be a proline instead of threonine. The other three mutations were in the nonstructural genes, two of which caused amino acid changes, and one that was a silent mutation.

A.

Genome Nucleotide Position	Nucleotide		Amino Acid		
	DENVax4-B expected	DENVax4-B observed	DENVax4-B expected	DENVax4-B	Position
416	G	G/A	Cys	Cys/Tyr	Capsid
8769	A	A/C	Gln	Gln/Pro	NS5

B.

Genome Nucleotide Position	Nucleotide		Amino Acid		
	DENVax4-C expected	DENVax4-C observed	DENVax4-C expected	DENVax4-C	Position
400	A	C	Thr	Pro	Capsid
4420	A	C	Ile	Leu	NS2B
8269	A	T	Met	Leu	NS5
9201	A	C	Leu	Leu	NS5

Table 3.9 **Genomic Sequencing of DENVax4-b and DENVax4- second generation viruses.**

3.5. DISCUSSION

For any multivalent vaccine candidate, the goal is to generate a balanced immune response *in vivo*. DENVax consists of chimeric dengue virus vaccines to each of the four serotypes of dengue. The immune response to the dengue-4 component of the tetravalent dengue vaccine is limited when compared to the other vaccine virus serotypes, which led to re-engineering the current DENVax4 vaccine. Three variants were designed to explore the contribution of sequences at the 5' junction of the chimera to replication of the recombinant virus. We successfully designed and generated the three variants. Phenotypic and genetic characterization of these variants was completed, including growth characteristics *in vitro*, plaque phenotype *in vitro*, and sequencing of the entire genome.

After electroporation, the DENVax4-d variant did not produce viable virus. This could be because the amino acid deletion that restored the serine to position C-100 prevented the virus from successfully growing in Vero cells. Viable DENVax4-b and DENVax4-c viruses were grown after electroporation and subsequent experiments were performed using only these two variants. Preliminary evidence suggests both the –b, and –c variants grow to equivalent titers of the original DENVax4 in Vero cells. Plaque size of the –b variant more closely mimics the original, and the –c variant has a heterogeneous plaque phenotype when visualized in Vero cells. For future experiments the DENVax4-c viruses should be re-titrated to plaque pick small and large plaques from the heterogeneous mixture. The different sized plaques can then be amplified, and the RNA purified and sequenced to compare the mutations in the small and large plaques.

Additionally, the growth characteristics in mosquito cells were different for the DENVax4-b and DENVax4-c viruses when compared to wild type dengue-4. Wild-type Dengue-4 grew to high titers (1.5×10^8 pfu/ml). Published studies characterizing the growth of the original DENVax4 candidate show that it is capable of limited growth in this mosquito cell line (3.16×10^4 pfu/ml) (*24*). Interestingly, the –b variant grew to a peak titer of 2.7×10^6 pfu/ml, which indicates some level of fitness in this cell line. The –c variant had no growth in C6/36 cells until after day 6 when, presumably, it mutated as it adapted to this cell line *in vitro* and grew to a titer of 2.2×10^4. Any effective and safe dengue vaccine would need to show reduced ability to grow in mosquito cells, to limit the small chance of transmission in nature. Although the C6/36 cells can give preliminary data about the replication efficiency of viruses in mosquitoes, transmission studies would need to be performed in live *Aedes aegypti* mosquitoes. Future studies to evaluate the immunogenicity of these vaccine strains are currently being planned to compare with DENVax4.

Another future experiment includes additional adaptation of the second-generation viruses in Vero cells. The viruses can be blind passaged ten times and plaque titrated for each passage to evaluate plaque size and phenotype. After the tenth passage the viruses will be sequenced and then analyzed. If there are any mutations that further adapt the viruses for growth in Vero cells they will be re-engineered into the cDNA infectious clones.

Phase 1 clinical trials in humans are beginning in the spring of 2010 to test the safety and immunogenicity of DENVax. To be included in the tetravalent formulation of any future

DENVax vaccine, a second generation DENVax4 must undergo rigorous *in vitro* scale-up and *in vivo* testing to assure safety and immunogenicity. These would include evaluating neurovirulence in mice, immunogenicity in mice and nonhuman primate, replication in live mosquitoes, and temperature sensitivity testing *in vitro*. Multiple animal studies in mice and NHP to compare the current DENVax4 to a second generation would be a prelude to a possible bridging study in humans.

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