Published in final edited form as:
J Med Virol. 2011 May ; 83(5): 910–920. doi:10.1002/jmv.22033.

Microarray Hybridization for Assessment of the Genetic Stability of Chimeric West Nile/Dengue 4 Virus

Majid Laassri[1], **Bella Bidzhieva**[1], **James Speicher**[2], **Alexander G. Pletnev**[2,*], and **Konstantin Chumakov**[1,*]

[1]Laboratory of Method Development, Center for Biologics Evaluation and Research, US Food and Drug Administration, 1401 Rockville Pike, HFM 470, Rockville, MD 20852, USA.

[2]Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892, USA.

Abstract

Genetic stability is an important characteristic of live viral vaccines because an accumulation of mutants can cause reversion to a virulent phenotype as well as a loss of immunogenic properties. This study was aimed at evaluating the genetic stability of a live attenuated West Nile (WN) virus vaccine candidate that was generated by replacing the pre-membrane and envelope protein genes of dengue 4 virus with those from WN. Chimeric virus was serially propagated in Vero, SH-SY5Y human neuroblastoma and HeLa cells and screened for point mutations using hybridization with microarrays of overlapping oligonucleotide probes covering the entire genome. The analysis revealed several spontaneous mutations that led to amino acid changes, most of which were located in the envelope (E) and non-structural NS4A, NS4B, and NS5 proteins. Viruses passaged in Vero and SH-SY5Y cells shared two common mutations: $G_{2337}C$ ($Met_{457}Ile$) in the E gene and $A_{6751}G$ ($Lys_{125}Arg$) in the NS4A gene. Quantitative assessment of the contents of these mutants in viral stocks indicated that they accumulated independently with different kinetics during propagation in cell cultures. Mutant viruses grew better in Vero cells compared to the parental virus, suggesting that they have a higher fitness. When tested in newborn mice, the cell culture-passaged viruses did not exhibit increased neurovirulence. The approach described in this paper could be useful for monitoring the molecular consistency and quality control of vaccine strains.

Keywords

Flavivirus vaccine; mutants screening; mutants quantitation; molecular consistency; quality control; West Nile Virus; vaccine; cell culture; oligonucleotide microarray; mutations screening; mutant quantitation

Introduction

West Nile (WN) virus is an enveloped positive-sense single-stranded RNA virus, which belongs to the Flavivirus genus in the *Flaviviridae* family [Lindenbach et al., 2007; Lindenbach and Rice, 2003]. It is a member of the Japanese encephalitis virus (JE) antigenic complex, a group of mosquito-borne flaviviruses that includes important human neurotropic pathogens such as the JE, Murray Valley encephalitis, and St. Louis encephalitis viruses [Burke and Monath, 2001]. The WN RNA genome is approximately 11 kb in length and

*Corresponding author: Konstantin Chumakov, CBER/FDA, 1401 Rockville Pike HFM-470, Rockville, MD 20852-1448, USA. Phone: (301) 594-3720, Fax: (301) 827-4622. konstantin.chumakov@fda.hhs.gov.. Co-corresponding author: Alexander G. Pletnev, apletnev@niaid.nih.gov.

contains a single open reading frame encoding a polyprotein that is co-translationally processed by viral and cellular proteases to form three structural (capsid [C], membrane precursor [prM], envelope [E]) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins. The structural proteins assemble with genomic RNA into mature virions and mediate virus attachment. They induce protective immunity in the host, while the non-structural proteins regulate viral RNA replication and translation and attenuate host antiviral responses (reviewed in [Diamond, 2009; Lindenbach et al., 2007; Robertson et al., 2009]).

WN is maintained in nature in a transmission cycle between mosquitoes and birds, while humans, horses, and other domestic and wild animals serve as incidental hosts. While a majority of human WN infections are asymptomatic, about 20% of individuals infected with WN display symptoms ranging from flu-like illness to severe neurological disease with a high mortality rate. The emergence of this virus in North America has resulted in a significant increase in the disease being observed in humans, horses, and birds. During the 1999-2009 outbreaks of WN in the USA, there were at least 28,961 reported human cases of WN illness that included 1,159 deaths (CDC Reports: http://www.cdc.gov/ncidod/dvbid/westnile/surv&control.htm#casedefinition). Importantly, the severe neurological disease (meningitis or encephalitis) that requires long-term rehabilitation was observed in over 30% of the confirmed WN cases and occurred with increased frequency in elderly and immuno-compromized patients. Also, human-to-human transmission by organ transplants, mother to child transmission, and blood transfusion have been reported (reviewed in [Petersen and Hayes, 2008]). Since there is no antiviral drug effective against WN infection, it is considered a significant public health threat. While there is a WN vaccine to protect domestic animals, no vaccine is licensed for use in humans, making the development of WN vaccines an important priority.

In an effort to develop a live attenuated vaccine against WN, a chimeric WN/DEN4Δ30 virus was previously generated by replacing the prM and E protein genes of the recombinant dengue type 4 virus that contains a 30 nucleotide deletion (nucleotides 10478-10507) in the 3′ non-coding region (DEN4Δ30) with the corresponding genes of a highly virulent WN strain [Durbin et al., 2001; Pletnev et al., 2002; Pletnev et al., 2006]. Preclinical studies in animal models confirmed the attenuation of WN/DEN4Δ30 in non-human primates and demonstrated a highly restricted replication in the central nervous system of mice, reduced infectivity for mosquitoes, and the absence of infectivity for birds, findings that indicate that this vaccine should be safe for both the recipient and the environment.

The genetic stability of live viral vaccines is a key element of their safety and protective efficacy. It must be studied as an important part of pre-licensure evaluation and quality control of live WN virus vaccine during its manufacture and after administration to vaccines. Even though WN/DEN4Δ30 chimera was produced from a cDNA clone, mutations rapidly emerge and could be selected during virus growth in cell culture [Pletnev et al., 2006]. Spontaneous mutations easily emerge in all single-strand RNA viruses; therefore, the consequences of their accumulation must be identified to ensure the safety of live vaccines. Because of the presence of a large number of mutants, populations of RNA viruses are often described as quasispecies. Most mutants are present at a relatively low level, making them difficult to detect using conventional sequencing methods. A new microarray-based approach was recently developed and used in this laboratory to screen for emerging mutations in attenuated polioviruses [Cherkasova et al., 2003]. The microarray for re-sequencing and sequence heterogeneity (MARSH) analysis is based on the hybridization of fluorescently-labeled cDNA produced from the virus genome with microarrays of oligonucleotide probes that are complementary to, and cover, the entire viral genome. Quantitative comparison of hybridization data produced for a serially passaged test sample

with the data for homogeneous reference DNA revealed mutations that emerge and accumulate during the replication of vaccine strains *in vitro* or *in vivo*. The present study was designed to create a similar high-throughput microarray hybridization method to study the genetic stability of the WN vaccine. The microarray hybridization was used to identify genomic loci, in which mutations consistently accumulated. The presence of these mutations increased viral fitness and boosted the replication of cell-culture passaged virus in Vero cells. The potential role of these mutations was studied by neurovirulence tests in highly sensitive suckling mice to demonstrate that they were adventitious, i.e., did not result in reversion of the attenuated phenotype. A similar approach could be used for evaluation of the genetic stability of other flavivirus vaccines.

Materials and Methods

Viruses and Plasmids

West Nile and dengue viruses are members of Flavivirus genus belonging to *Flaviviridae* family of RNA-containing viruses. The WN/DEN4-3'Δ30 clinical lot virus [Pletnev et al., 2006] that will hereafter be referred to as the lot 1 virus was derived from a recombinant cDNA genome containing prM and E protein genes from WN strain NY99 and the rest of the genome sequence derived from dengue type 4 virus (DEN4), with a 30 nucleotide deletion in the 3′ non-coding region [Pletnev et al., 2002; Pletnev et al., 2006]. Sequence analysis of the virus derived from WN/DEN4-3'Δ30 recombinant plasmids revealed that during its recovery from DNA and 6-passage amplification in Vero cells, it accumulated five nucleotide changes (Table 1), four of which produced amino acid substitutions in the E, NS2A, NS3, and NS4B proteins. Throughout this paper numbering of nucleotide and amino acid sequences of the prM and E protein genes were derived from the sequence of WN NY99 (GenBank accession number AF196835) and numbering of nucleotide and amino acid sequences of nonstructural protein genes were derived from the sequence of DEN4 p4D30 (GenBank accession number AY376438). To produce a virus that matched the genomic sequence of this virus, the plasmid WN/DEN4-3'Δ30 clone 1 DNA, from which lot 1 was derived, was modified by site-directed mutagenesis. Four coding changes were introduced into WN/DEN4-3'Δ30 clone 1: $Gly_{314}Arg$ in E, $Gln_{2}Pro$ in NS2A, and $Thr_{102}Ala$ and $Val_{216}Ala$ in NS4B. The virus was recovered in qualified Vero cells by transfection with purified RNA transcripts produced from the linearized newly generated plasmid DNA, designated pWN/DEN4Δ30-SW #134. The recovered virus was grown in qualified Vero cells, biologically cloned by three passages at terminal dilution, and finally amplified by two passages in Vero cells. The final product seed virus , the WN/DEN4Δ30#108A lot of chimeric WN/DEN4-3'Δ30 virus (referred to as clinical lot 2), had totally 6 passages in qualified Vero cells and attained a titer of 2×10^7 pfu/ml. Sequence analysis of the clinical lot 2 virus revealed that its genome contained no mutations except those that were engineered into the plasmid DNA. Table 1 lists the nucleotide and amino acid changes observed in clinical lots 1 and 2, and shows that there were no additional amino acid differences between the two lots in the polyprotein or nucleotide changes in the 5' or 3' NCRs. The only difference was a single silent mutation, $A_{4550}C$ in the NS3 gene of clinical lot 1, and the presence of the engineered silent mutations in clinical lot 2.

The plasmid DNA (pWN/DEN4Δ30-SW #134), from which lot 2 was derived, was used as a reference for the microarray assay. Two other plasmid cDNAs of WN/DEN4-3'Δ30 (designated as clone P1 [Pletnev et al., 2006] and clone P1-1 that contained the engineered $T_{4891}C$ ($Ile_{123}Thr$) mutation in the NS3 protein gene [Blaney et al., 2002]) were used for validation of the microarray assay.

Virus growth in cell cultures

To study the genetic stability of lot 1 virus, its three independent lineages were serially passaged 12 times in Vero cells (continuous monkey kidney cells, World Health Organization master cell bank, passages 143 to 150) infected with a multiplicity of infection (MOI) of 1.0 and maintained at 37 °C in an atmosphere of 5% CO_2 in minimal essential medium (MEM) (Invitrogen) supplemented with heat-inactivated 10% fetal bovine serum (FBS; Cambrex, Walkersville, MD), 2 mM L-glutamine (Invitrogen), and 0.05 mg/ml of gentamicin (Invitrogen). After incubation at 37 °C for 6 days, the cell culture medium was harvested, clarified by centrifugation, assayed for virus titer, and then used for the next virus passage. The virus titer was determined by titration in Vero cells using a plaque-forming assay described previously [Pletnev et al., 2006]. Lot 2 virus was also passaged in Vero, HeLa, MDCK (dog kidney), and UMNSAH/DF-1 (chicken embryo fibroblast) cells as described above, and in human SH-SY5Y neuroblastoma cells that were maintained in Dulbecco's modified Eagle F12 medium (DMEM/F12, Invitrogen) supplemented with 10% FBS and 0.05 mg/ml of gentamicin. SH-SY5Y, HeLa and MDCK cells were obtained from the Cell Bank of the Laboratory Method Developments (CBER/FDA), and UMNSAH/DF-1 cells were purchased from the American Type Culture Collection.

Virus quantitation by qRT-PCR

To monitor viral replication in cell cultures, the amount of harvested viruses was determined by quantitative RT-PCR. RNA from 140 μl of virus-containing medium was isolated using a QIAamp Viral RNA Mini Kit (QIAGEN). The RNA was eluted in a final volume of 60 μl of sterile RNase-free water.

The extracted RNA from the harvested viruses was quantified by a one-step quantitative RT-PCR assay using the QuantiTect SYBR Green RT-PCR kit (QIAGEN). RT-PCR was performed in 50 μl reactions containing 3 μl of template RNA, 25 μl of 2XQuantiTect SYBR Green RT-PCR Master Mix, 0.5 μl of QuantiTect RT Mix and 0.4 μM of the forward 504WND4TaqF primer 5'-GACGTCACAGATGTCATCACGAT and the reverse 578WND4TaqR primer 5'-TCCCACATCCATTGCTCTGA. These primers were designed to produce the PCR-fragment containing nucleotides 504-578 of the WN/DEN4Δ30 genome. Quantitative RT-PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems). The RT-PCR protocol included a reverse transcription for 30 min at 50 °C and cDNA denaturation for 15 min at 95 °C, followed by 40 cycles of cDNA PCR amplification. Each cycle consisted of a denaturation step for 15 sec at 94 °C, annealing for 30 sec at 52 °C, and an extension step for 30 sec at 72 °C. The standard curve was generated using RNA extracted from Lot 2 virus ($2.5\ 10^7$ pfu/ml) as a standard. A 10-fold dilution series of viral RNA (from 10^{-2} to 10^{-5}) were used to produce a standard curve, from which the viral RNA test quantities were determined. All reactions were performed in duplicate with negative (water) and positive controls (known RNA). In preliminary experiments, qRT-PCR was calibrated using virus stocks with known titers. This allowed us to express virus content as PFU/ml.

Evaluation of WN/DEN4Δ30 viruses for neurovirulence in newborn mice

Mice were handled according to the Federal and NIAID Animal Care and Use Committee regulations. To determine the neurovirulence of parental WN/DEN4Δ30 (lots 1 and 2) virus and its mutants derived by cell-culture passaging, 3-day-old Swiss Webster mice (Taconic Farms, Germantown, NY) in litters of 10 were inoculated via the intracerebral (IC) route with 10-fold dilutions of the virus at doses ranging from 10^2 to 10^4 PFU/mouse. The mice were monitored for morbidity and mortality for up to 21 days post-inoculation (dpi). Moribund (i.e., paralyzed) mice were humanely euthanized and scored as a lethality. The IC 50% lethal dose (IC LD_{50}) was determined by the Reed & Muench method [Reed, 1938].

Design of PCR primers, oligoprobes, and microarrays

The nucleotide sequence heterogeneity of WN/DEN4Δ30 was assessed using a microarray hybridization method similar to the one described previously for poliovirus [Cherkasova et al., 2003] (See scheme shown on Fig. 1). Viral cDNA was prepared with reverse transcriptase SuperScript III (Invitrogen) using specific reverse primer WN10651RT7 (Table 2). Microarrays of immobilized oligonucleotide probes were hybridized with fluorescently-labeled RNA transcribed by T7 RNA polymerase from PCR-amplified viral cDNA. The entire WN/DEN4Δ30 genome was amplified using the specific primers listed in Table 2 to produce 8 overlapping DNA segments (Fig. 1). RNA for hybridization was produced by *in vitro* transcription of the PCR products with a MEGAscript T7 RNA polymerase kit (Ambion Inc.). 10 μg of each RNA product were fluorescently labeled with a Cy3 Micromax ASAP RNA Labeling Kit (Perkin-Elmer). Labeled RNA samples were purified using CENTRI-SEP (Princeton Separation) spin columns. Hybridization between microarray oligoprobes and fluorescently-labeled RNA was performed using 8 microarrays corresponding to overlapping parts of the WN/DEN4Δ30 genome PCR-amplified from cDNA. A total of 1096 oligoprobes (16-25 nucleotides long) with melting temperatures around 50 °C were synthesized to cover the entire genome of the WN/DEN4Δ30 lot 2 virus and to overlap each other at half-length.

Oligonucleotide probes were amino-modified during synthesis [Wade et al., 2004] to enable covalent attachment to CodeLink Activated (CLA) slides (CodeLink, Amersham Biosciences) using a contact micro-spotting robot PIXSYS 5500 (Cartesian Technologies, Inc.). Each microarray contained four identical sub-arrays that were hybridized together to assess reproducibility of the hybridization results and to eliminate outlier data points. Hybridization signals from individual sub-array elements that differed from the average value calculated for all 4 replicates of the oligoprobe by more than 2 standard deviations were discarded. The number of invalidated data points did not exceed 0.1%. Next, average values from four sub-arrays were normalized by the total fluorescence signal from the entire array. Finally, normalized signals from the reference sample (homogeneous DNA) were divided by the respective normalized signals from the test samples, and the results were expressed as a ratio. Regions with no mutations produced ratios close to 1, while mutations in test samples led to reduced hybridization with some oligoprobes, and therefore produced ratios greater than 1.

Microarray hybridization, scanning and data analysis

The microarray hybridization was performed as previously described [Cherkasova et al., 2003; Laassri et al., 2005; Laassri et al., 2007]. Briefly, fluorescently-labeled RNA samples were vacuum-dried prior to hybridization, reconstituted in ASAP Hybridization Buffer III (Perkin-Elmer), and denatured by incubation for 1 min at 95 °C. The final concentration of each probe in the hybridization solution did not exceed 0.1 μM. A five-microliter aliquot of the hybridization mixture was applied to the microarray area and covered with an individual plastic cover slip. Hybridization was performed in an incubation chamber (ArrayIt, CA) for 1 h at 45 °C. The fluorescent images of processed microarray slides were captured using ScanArray 5000 (PerkinElmer), and the images were analyzed using ScanArray Express software (PerkinElmer).

Sequence analysis

To validate the results of microarray analysis, the complete genome consensus sequences of the high-passage virus stocks of lot 1 virus passaged in Vero cells and lot 2 viruses passaged in Vero, HeLa and SH-SY5Y human neuroblastoma cells were determined. The sequencing was performed in two directions using the ABI PRISM BigDye Terminator v3.1 Cycle

Sequencing Kit's protocol (Applied Biosystems) and an ABI Prism 310 genetic analyzer system (PE Applied Biosystems).

MAPREC assay

Quantitation of mutants was performed by mutant analysis by PCR and restriction enzyme cleavage (MAPREC) as previously described [Chumakov et al., 1992; Chumakov et al., 1991] with some modifications. Briefly, viral RNA was extracted and reverse transcribed into cDNA (see above). A short DNA segment containing the mutation of interest was PCR-amplified using partially mismatched primers. The mismatches were located near the 3'-end of the primers and created or destroyed restriction sites that included the mutation of interest (Table 3). The quantity of mutants in a sample was determined by the proportion of PCR-amplified DNA labeled with IRDye700 near-infrared dye that was cleaved by the appropriate restriction endonuclease. Restriction enzyme digestion products were separated by electrophoresis in 10% native polyacrylamide gels. The gel image capture and band quantifications were carried out using an Odyssey infrared imaging system and Odyssey v3.0 software (LI-COR Biosciences).

Results

Design and validation of the oligonucleotide microarray

To detect sequence heterogeneities in the genome of WN recombinant WN/DEN4Δ30 virus, 1096 oligonucleotide probes that covered the entire genome of the virus were synthesized. They had the melting temperatures around 50 °C (16-25 nucleotides in length). Adjacent oligonucleotide probes overlapped each other at half-length. The entire set of oligoprobes was printed on eight individual microarrays corresponding to parts of the virus genome that were amplified by PCR (Fig. 1A). Each microarray consisted of four identical sub-arrays that were hybridized together to ensure reproducibility of the microarray results. Viral genome was amplified in eight PCR amplicons (corresponding to the eight microarrays described above) using the primers listed in Table 2. The reverse primer in each primer pair contained a T7 RNA polymerase promoter that was used to generate fluorescently labeled RNA transcripts. The RNA of the test samples was hybridized with microarrays with control RNA transcripts derived from a plasmid DNA that was used to generate the WN/DEN4Δ30 Lot 2 virus (Fig. 1B). The results of the microarray analysis were expressed as the ratio of hybridization intensities produced by each microarray element hybridized with this homogeneous reference sequence versus the hybridization intensities obtained with a test sample (see Materials and Methods). The peaks on these plots indicated the presence of mutation(s) that disrupted hybridization with oligoprobes on the microarray (Fig. 1C). The ability of this method to detect mutations in WN recombinant WN/DEN4Δ30 genomes was demonstrated in pilot experiments by testing plasmid samples containing known mutations at defined sites of the chimeric genome (data not shown).

Analysis of the genetic stability of WN/DEN4Δ30 virus propagated in different cell lines

To study the genetic stability of WN recombinants (WN/DEN4Δ30), they were propagated in different cell lines and harvested on day 6 after infection, when the peak infectivity was reached. WN/DEN4Δ30 Lot 2 virus was serially passaged in Vero cell culture certified for use in vaccine production and screened for mutations that may have accumulated during passaging. In order to get additional information about its genetic stability and to reveal potentially unstable genomic loci, WN/DEN4Δ30 Lot 2 virus was passaged in other cell cultures that can be used for virus vaccine manufacture (dog kidney MDCK and chicken embryo fibroblast UMNSAH/DF-1 cells) or had a human origin (HeLa). In addition, vaccine virus was tested in SH-SY5Y human neuroblastoma cells to reveal mutations that could accumulate during replication in cells of neuronal origin and potentially increase its

neurovirulence. Microarray analysis of the viruses passaged in Vero, SH-SY5Y, and HeLa cells revealed several peaks indicating the presence of mutations (Fig. 2). Genomic regions containing these peaks were then subjected to nucleotide sequence analysis (Table 4). Most mutations were detected in the E gene and one mutation was detected in each of the NS4A and NS4B genes. Two mutations (Met_{457}Ile in the E gene and Lys_{125}Arg in the NS4A gene) were detected in both SH-SY5Y and Vero cell-passaged viruses (Table 4). A similar analysis was performed for the WN/DEN4Δ30 Lot 1 virus that was passaged 12 times in Vero cells. It revealed one additional mutation (Asn_{390}Lys) in the NS5 gene. No peaks in the microarray ratio plots were detected in the C, prM, NS1, NS2A, NS2B, or NS3 genes. Sequencing analysis of these genes also did not reveal any mutations in high-passage viral stocks.

The kinetics of accumulation of the WN/DEN4Δ30 mutants in cell cultures

To determine the rate of accumulation of the mutants discovered in high-passage stocks of WN/DEN4Δ30 virus, they were quantified in each passage using a modified MAPREC assay with near infrared IRDye-700 dye instead of a radioactive label (see Materials and Methods). Analysis of C_{1285}T, A_{1657}G, and T_{7153}C mutations identified in virus passaged in HeLa cells revealed that only T_{7153}C mutants were present in the unpassaged Lot 2 virus, albeit at a very low level (1.6%). The results presented in Fig. 3 show that these mutations gradually accumulated and reached the highest content by passage 4 in HeLa cells. G_{2337}C and A_{6751}G mutants detected in both Vero and neuroblastoma cell passages could be detected in the first passage and gradually accumulated to reach their highest content of 85% and 91% by passage 7 in Vero cells, and 79% and 42% by passage 6 in SH-SY5Y cells, respectively. G_{2337}C mutants were present in the unpassaged Lot 2 virus at about the 30% level. The G_{1111}A mutants detected only in SH-SY5Y passaged virus accumulated to 69% by passage 6. The different kinetics of accumulation of all of these mutants suggested that they were selected independently by providing replicative advantage to the virus.

Growth kinetics of the Lot 2 virus mutants

Gradual accumulation of these mutations suggested that they increased viral fitness and may improve its replication. To test this, viral replication in Vero cells was studied by using quantitative RT-PCR (Fig. 4). Virus that contained the Met_{457}Ile mutation in the E gene and Lys_{125}Arg mutation in the NS4A gene grew in SH-SY5Y and Vero cells to levels more than 10-fold higher than in parental virus (Fig. 4A). In addition, the kinetics of replication of the mutant virus was compared by infecting Vero cells at a MOI of 0.1 pfu/cell and measuring virus yield at one day intervals over a 13 day period (Fig. 4B). The results showed that the viruses that contained the Met_{457}Ile mutation in the E gene and the Lys_{125}Arg mutation in the NS4A gene replicated more efficiently in Vero cells, with an estimated virus concentration reaching more than 9 $\log_{10}$ pfu/ml on day 8 post-infection compared to their parental virus, which reached only 8 $\log_{10}$ pfu/ml on day 8 post-infection.

Analysis of neurovirulence in newborn mice

Adaptation of the WN/DEN4Δ30 virus to different cell cultures resulted in higher virus growth fitness, which could contribute differently to the pathogenic phenotype of this live attenuated virus vaccine candidate. To evaluate the neurovirulence of serially passaged WN/DEN4Δ30 viruses, the morbidity and mortality of 3-day-old mice after intracerebral (IC) inoculation were determined. Mice of this age were selected since they are the most sensitive animal model for evaluation of the pathogenicity of neurotropic flaviviruses or their live attenuated vaccines. WN was previously shown to be highly neurovirulent in suckling mice with an IC LD_{50} of 0.4 PFU [Pletnev et al., 2002]. In the present study, two clinical lots of WN/DEN4Δ30 were evaluated in parallel by IC inoculation of a virus dose ranging from 10^2 to 10^4 PFU and using morbidity and mortality as the endpoints. During the

21-day observation period, only 1 of 10 mice died when inoculated with 10^2 PFU of Lot 1 virus or 10^3 PFU of Lot 2 virus (data not shown). Consistent with our previous observations [Pletnev et al., 2002; Pletnev et al., 2006], these results indicated that WN/DEN4Δ30 virus retained its attenuated phenotype, as it was highly attenuated in newborn mice compared to its WN parent. Next, the clinical Lot 2 virus was compared with its cell culture-passaged derivatives using the same test (Fig 5). One or two of the 10 suckling mice died when inoculated with 10^2 or 10^4 PFU of parental or mutant virus, demonstrating that despite the genetic differences between these viruses, there was no significant increase in the level of neurovirulence of strains passaged in Vero and SH-SY5Y cells. Therefore, both chimerization between WN and DEN4 and the presence of Δ30 deletions, but not the accumulation of the adventitious cell culture-adapting mutations, are the major genetic determinants of neurovirulence attenuation of WN/DEN4Δ30 virus for mice.

Discussion

Live viral vaccines are produced from attenuated strains that are generated by either adaptation to growth in non-natural cell substrates or by genetic manipulations that reduce virus fitness. In both cases, attenuated strains are crippled in their ability to replicate in target organs, but are still able to induce a protective immune response. Virus growth during vaccine manufacture and replication in vaccine recipients can result in the accumulation of adventitious mutants that increase viral fitness and can change its virulence. In addition, mutations can alter antigenic structure and thereby reduce vaccine efficacy. Therefore, genetic and phenotypic stability is an important property of live viral vaccines. This is especially true for RNA viruses whose RNA replicases have notoriously low fidelity, and therefore their populations consist of a mixture of mutants frequently described as quasispecies. One dose of many RNA virus vaccines contains all possible mutants, some of which can accumulate in a population if they provide replicative advantage, or become dominant in the virus population because of random selection due to bottlenecking. Therefore, it is critically important to assess the genetic stability of new strains proposed for the production of live viral vaccines.

Genome chimerization is an effective way to attenuate flaviviruses and has been used to create a number of prospective vaccines [Guirakhoo et al., 2004; Lindenbach et al., 2007; Lindenbach and Rice, 2003; Monath et al., 1999; Pletnev et al., 2001; Pletnev et al., 1992; Pletnev et al., 2000; Pletnev and Men, 1998; Pletnev et al., 2002; Pletnev et al., 2006]. Incomplete match between heterologous parts of chimeric genomes probably plays an important role in the reduction of viral fitness that leads to attenuation. This creates a selective pressure for the accumulation of mutants that restore virus fitness by adapting heterologous genomic parts to each other and could lead to a loss of attenuation. Accumulation of mutations and genetic instability of flaviviruses were reported previously. For instance, [Dunster et al., 1999] showed that passaging of wild Yellow Fever virus (YFV) in HeLa cells leads to its attenuation and accumulation of mutations. Previous studies of genetic stability of flavivirus chimeras based on YFV performed by virus passaging and conventional nucleotide sequencing also demonstrated that mutations accumulate during virus passaging [Pugachev et al., 2004; Pugachev et al., 2002; Pugachev et al., 2007], confirming the need to identify genomic loci that are prone to the accumulating mutants and to determine their phenotypes. If mutations in unstable genomic loci increase virulence, than methods to prevent their emergence and control their presence in vaccine preparations could be developed. On the other hand, if the fitness-restoring mutants do not lead to de-attenuation, then it may be desirable to incorporate them into the genetic makeup of the vaccine strain. Additionally, increased yields of such viruses during vaccine production may help stabilize the genome by relieving selective pressure, thereby preventing random and

potentially undesirable mutations from being passively selected through the “passenger effect”.

The most straightforward way to assess genetic stability is to passage the virus under conditions of vaccine production, and then analyze phenotypes and screen for mutations that may have accumulated. The biggest challenge in this process is a sensitive screening for mutations because conventional sequencing methods do not allow for the detection of mutations if they are present at levels below 20-30% of the population. Recently, a new method based on hybridization with microarrays of immobilized short oligonucleotides was proposed in this laboratory [Cherkasova et al., 2003]. These microarrays allowed us to detect mutants in samples of serially passaged poliovirus before they could be identified by sequencing [Cherkasova et al., 2003]. Later, this approach was used to look for sequence heterogeneity in other systems [Laassri et al., 2005; Laassri et al., 2007].

In this report, the genetic stability of WN/DEN4Δ30 chimeras [Pletnev et al., 2003; Pletnev et al., 2002; Pletnev et al., 2006] was analyzed by serially propagating WN/DEN4-3’Δ30 Lot 1 virus in Vero cells using three independent lineages. This virus was highly stable in Vero cells, as only one mutation was detected in the NS5 gene ($Asn_{390}Lys$) after 12 consecutive passages. We also analyzed a derivative virus (WN/DEN4-3’Δ30#108A Master seed virus referred to as Lot 2) that was proposed for vaccine production. This virus contained several additional mutations that were introduced to adapt it to better growth in Vero cells. To identify potentially unstable genomic loci, the WN/DEN4Δ30 Lot 2 virus was serially passaged in Vero cells as well as in SH-SY5Y human neuroblastoma cells to reveal possible mutations that adapt the virus to growth in neuronal tissues. It had been also passaged in HeLa, MDCK (dog kidney), and DF-1 (chicken fibroblast) cells as a control. The virus replicated very poorly in MDCK and DF-1 cells, and propagation had to be stopped after four passages. Growth in Vero and SH-SY5Y cells led to virus adaptation, as evidenced by higher titers reached by passaged virus. The growth of the HeLa cell-passaged virus was similar to the original strain.

To identify the mutations responsible for adaptation, the passaged virus stocks were subjected to mutational screening using the MARSH procedure. Two mutations were found in the virus propagated in Vero cells, three mutations in SH-SY5Y cells, and four mutations in HeLa cells. All mutations led to amino acid changes except for one silent $C_{1285}T$ mutation. Analysis of emergence of these mutants performed by their quantitation in passages using the MAPREC assay showed that the kinetics of their accumulation was different, suggesting that they were likely selected independently because of selective pressure, rather than by random selection due to bottlenecking. Stocks grown in Vero and SH-SY5Y cells shared two common mutations, $G_{2337}C$ ($Met_{457}Ile$) in the E gene and $A_{6751}G$ ($Lys_{125}Arg$) in the NS4A gene. The $Met_{457}Ile$ mutation was located in the non-conservative stem-anchor region of the envelope protein that is believed to be a trans-membrane hydrophobic domain that serves as an endoplasmic reticulum membrane anchor to E [Zhang et al., 2003].

Virus passaged in HeLa cells contained a $Glu_{390}Lys$ mutation in domain III of the E protein, as well as one other mutation of $A_{1657}G$, ($Thr_{231}Ala$) located in the dimerization domain II of the E protein [Allison et al., 2001; Nybakken et al., 2006], which undergoes conformational change during the membrane fusion process, making it possible that this mutation can affect the process.

Testing of the cell culture-passaged mutant viruses in highly sensitive newborn mice showed that there was no major increase in neurovirulence, suggesting that the cell culture adaptation is not linked to a loss of attenuation phenotype. The chimerization between WN

and DEN4 and the Δ30 deletion mutation, but not the presence of the adventitious cell culture-adaptation mutations, were the major genetic determinants of attenuation of WN/DEN4Δ30 virus for neurovirulence in suckling mice. The practical implication of our findings could be two-fold. First, quantitation of these mutations in virus stocks prepared in the course of vaccine manufacture could be used for monitoring the consistency of vaccine production, similar to what is being done for Oral Poliovirus Vaccine [WHO, 2002]. Second, since the mutations that accumulated in the course of virus adaptation to cell cultures resulted in better growth but did not increase neurovirulence as measured in newborn mice, their incorporation could improve the vaccine strain and increase virus yield during vaccine manufacture.

Acknowledgments

We thank Dusan Blaskovic for his assistance in some microarray experiments, Maria Rios and Andriyan Grinev for kindly providing us with the RNA of WN-NY99 strain, and Drs. Joseph Blaney and Stephen Whitehead (NIAID) for generation and kind provision of the clinical lot 2 WN/DEN4Δ30 virus. This study was supported through the Interagency Agreement 224-06-1322/Y1-AI-6153-01 between FDA and NIH and by funds from the NIAID Division of Intramural Research.

References

Allison SL, Schalich J, Stiasny K, Mandl CW, Heinz FX. Mutational evidence for an internal fusion peptide in flavivirus envelope protein E. J Virol. 2001; 75(9):4268–4275. [PubMed: 11287576]

Blaney JE Jr. Johnson DH, Manipon GG, Firestone CY, Hanson CT, Murphy BR, Whitehead SS. Genetic basis of attenuation of dengue virus type 4 small plaque mutants with restricted replication in suckling mice and in SCID mice transplanted with human liver cells. Virology. 2002; 300(1): 125–139. [PubMed: 12202213]

Burke, DC.; Monath, TP. Flaviviruses. Knipe, DM.; Howley, PM., editors. Philadelphia: 2001. p. 1043-11251.

Cherkasova E, Laassri M, Chizhikov V, Korotkova E, Dragunsky E, Agol VI, Chumakov K. Microarray analysis of evolution of RNA viruses: evidence of circulation of virulent highly divergent vaccine-derived polioviruses. Proc Natl Acad Sci U S A. 2003; 100(16):9398–9403. [PubMed: 12878723]

Chumakov KM, Norwood LP, Parker ML, Dragunsky EM, Ran YX, Levenbook IS. RNA sequence variants in live poliovirus vaccine and their relation to neurovirulence. J Virol. 1992; 66(2):966–970. [PubMed: 1309923]

Chumakov KM, Powers LB, Noonan KE, Roninson IB, Levenbook IS. Correlation between amount of virus with altered nucleotide sequence and the monkey test for acceptability of oral poliovirus vaccine. Proc Natl Acad Sci U S A. 1991; 88(1):199–203. [PubMed: 1846038]

Diamond MS. Mechanisms of evasion of the type I interferon antiviral response by flaviviruses. J Interferon Cytokine Res. 2009; 29(9):521–530. [PubMed: 19694536]

Dunster LM, Wang H, Ryman KD, Miller BR, Watowich SJ, Minor PD, Barrett AD. Molecular and biological changes associated with HeLa cell attenuation of wild-type yellow fever virus. Virology. 1999; 261(2):309–318. [PubMed: 10497116]

Durbin AP, Karron RA, Sun W, Vaughn DW, Reynolds MJ, Perreault JR, Thumar B, Men R, Lai CJ, Elkins WR, Chanock RM, Murphy BR, Whitehead SS. Attenuation and immunogenicity in humans of a live dengue virus type-4 vaccine candidate with a 30 nucleotide deletion in its 3'-untranslated region. Am J Trop Med Hyg. 2001; 65(5):405–413. [PubMed: 11716091]

Guirakhoo F, Pugachev K, Zhang Z, Myers G, Levenbook I, Draper K, Lang J, Ocran S, Mitchell F, Parsons M, Brown N, Brandler S, Fournier C, Barrere B, Rizvi F, Travassos A, Nichols R, Trent D, Monath T. Safety and efficacy of chimeric yellow Fever-dengue virus tetravalent vaccine formulations in nonhuman primates. J Virol. 2004; 78(9):4761–4775. [PubMed: 15078958]

Laassri M, Dragunsky E, Enterline J, Eremeeva T, Ivanova O, Lottenbach K, Belshe R, Chumakov K. Genomic analysis of vaccine-derived poliovirus strains in stool specimens by combination of full-

length PCR and oligonucleotide microarray hybridization. J Clin Microbiol. 2005; 43(6):2886–2894. [PubMed: 15956413]

Laassri M, Meseda CA, Williams O, Merchlinsky M, Weir JP, Chumakov K. Microarray assay for evaluation of the genetic stability of modified vaccinia virus Ankara B5R gene. J Med Virol. 2007; 79(6):791–802. [PubMed: 17457926]

Lindenbach, B.; Thiel, H.; Rice, C. Flaviviridae: The viruses and their replication.. In: Knipe, D.; Howley, P., editors. Fields Virology. 5th edition. Lippincott-Raven Publishers; Philadelphia: 2007. p. 1101-1152.

Lindenbach BD, Rice CM. Molecular biology of flaviviruses. Adv Virus Res. 2003; 59:23–61. [PubMed: 14696326]

Monath TP, Soike K, Levenbook I, Zhang ZX, Arroyo J, Delagrave S, Myers G, Barrett AD, Shope RE, Ratterree M, Chambers TJ, Guirakhoo F. Recombinant, chimaeric live, attenuated vaccine (ChimeriVax) incorporating the envelope genes of Japanese encephalitis (SA14-14-2) virus and the capsid and nonstructural genes of yellow fever (17D) virus is safe, immunogenic and protective in non-human primates. Vaccine. 1999; 17(15-16):1869–1882. [PubMed: 10217584]

Nybakken GE, Nelson CA, Chen BR, Diamond MS, Fremont DH. Crystal structure of the West Nile virus envelope glycoprotein. J Virol. 2006; 80(23):11467–11474. [PubMed: 16987985]

Petersen LR, Hayes EB. West Nile Virus in the Americas. Medical Clinics of North America. 2008; 92(6):1307–1322. [PubMed: 19145778]

Pletnev AG, Bray M, Hanley KA, Speicher J, Elkins R. Tick-borne Langat/mosquito-borne dengue flavivirus chimera, a candidate live attenuated vaccine for protection against disease caused by members of the tick-borne encephalitis virus complex: evaluation in rhesus monkeys and in mosquitoes. J Virol. 2001; 75(17):8259–8267. [PubMed: 11483771]

Pletnev AG, Bray M, Huggins J, Lai CJ. Construction and characterization of chimeric tick-borne encephalitis/dengue type 4 viruses. Proc Natl Acad Sci U S A. 1992; 89(21):10532–10536. [PubMed: 1438242]

Pletnev AG, Claire MS, Elkins R, Speicher J, Murphy BR, Chanock RM. Molecularly engineered live-attenuated chimeric West Nile/dengue virus vaccines protect rhesus monkeys from West Nile virus. Virology. 2003; 314(1):190–195. [PubMed: 14517072]

Pletnev AG, Karganova GG, Dzhivanyan TI, Lashkevich VA, Bray M. Chimeric Langat/Dengue viruses protect mice from heterologous challenge with the highly virulent strains of tick-borne encephalitis virus. Virology. 2000; 274(1):26–31. [PubMed: 10936085]

Pletnev AG, Men R. Attenuation of the Langat tick-borne flavivirus by chimerization with mosquito-borne flavivirus dengue type 4. Proc Natl Acad Sci U S A. 1998; 95(4):1746–1751. [PubMed: 9465088]

Pletnev AG, Putnak R, Speicher J, Wagar EJ, Vaughn DW. West Nile virus/dengue type 4 virus chimeras that are reduced in neurovirulence and peripheral virulence without loss of immunogenicity or protective efficacy. Proc Natl Acad Sci U S A. 2002; 99(5):3036–3041. [PubMed: 11880643]

Pletnev AG, Swayne DE, Speicher J, Rumyantsev AA, Murphy BR. Chimeric West Nile/dengue virus vaccine candidate: preclinical evaluation in mice, geese and monkeys for safety and immunogenicity. Vaccine. 2006; 24(40-41):6392–6404. [PubMed: 16831498]

Pugachev KV, Guirakhoo F, Ocran SW, Mitchell F, Parsons M, Penal C, Girakhoo S, Pougatcheva SO, Arroyo J, Trent DW, Monath TP. High fidelity of yellow fever virus RNA polymerase. J Virol. 2004; 78(2):1032–1038. [PubMed: 14694136]

Pugachev KV, Ocran SW, Guirakhoo F, Furby D, Monath TP. Heterogeneous nature of the genome of the ARILVAX yellow fever 17D vaccine revealed by consensus sequencing. Vaccine. 2002; 20(7-8):996–999. [PubMed: 11803058]

Pugachev KV, Schwaiger J, Brown N, Zhang ZX, Catalan J, Mitchell FS, Ocran SW, Rumyantsev AA, Khromykh AA, Monath TP, Guirakhoo F. Construction and biological characterization of artificial recombinants between a wild type flavivirus (Kunjin) and a live chimeric flavivirus vaccine (ChimeriVax-JE). Vaccine. 2007; 25(37-38):6661–6671. [PubMed: 17693000]

Reed SC. Uniovular Twins in Mice. Science. 1938; 88(2270):13. [PubMed: 17743673]

Robertson SJ, Mitzel DN, Taylor RT, Best SM, Bloom ME. Tick-borne flaviviruses: dissecting host immune responses and virus countermeasures. Immunol Res. 2009; 43(1-3):172–186. [PubMed: 18841330]

Wade MM, Volokhov D, Peredelchuk M, Chizhikov V, Zhang Y. Accurate mapping of mutations of pyrazinamide-resistant Mycobacterium tuberculosis strains with a scanning-frame oligonucleotide microarray. Diagn Microbiol Infect Dis. 2004; 49(2):89–97. [PubMed: 15183857]

WHO O. Recommendations for the production and control of poliomyelitis vaccine (oral). WHO; 2002.

Zhang W, Chipman PR, Corver J, Johnson PR, Zhang Y, Mukhopadhyay S, Baker TS, Strauss JH, Rossmann MG, Kuhn RJ. Visualization of membrane protein domains by cryo-electron microscopy of dengue virus. Nat Struct Biol. 2003; 10(11):907–912. [PubMed: 14528291]

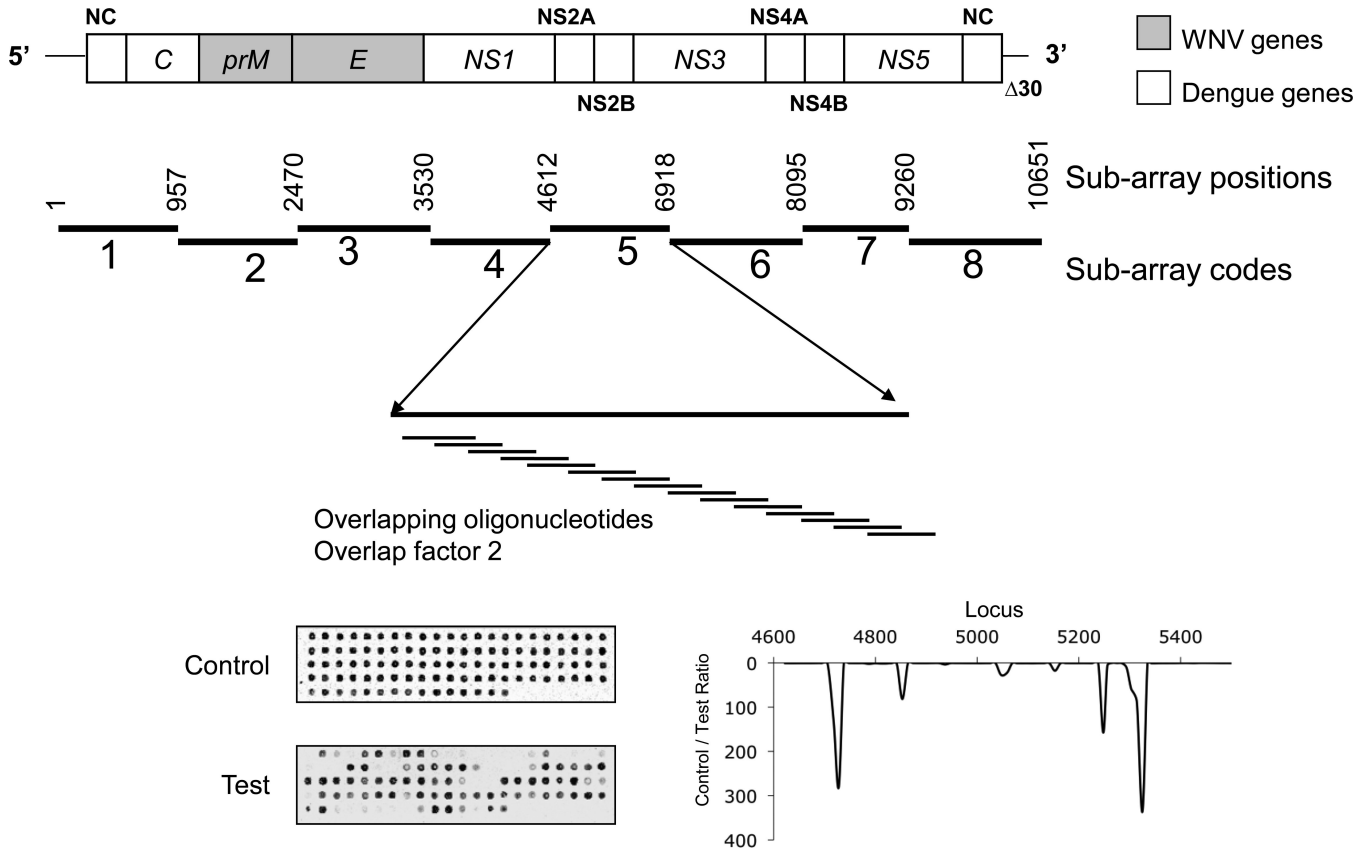

Figure 1.

Schematic overview of the MARSH assay: (A) Eight microarrays contained short oligonucleotides overlapped at half-length that covered the entire genome of the WN/DEN4Δ30 Lot 2 virus. Each microarray contained four identical sub-arrays that were hybridized together. (B) Images of individual sub-arrays hybridized with control and test samples. (C) Ratio of hybridization signals from reference and test sample preparations were plotted to reveal peaks indicating the presence of mutations. The hybridization images and the ratio plot are cartoons given for illustration purposes only.

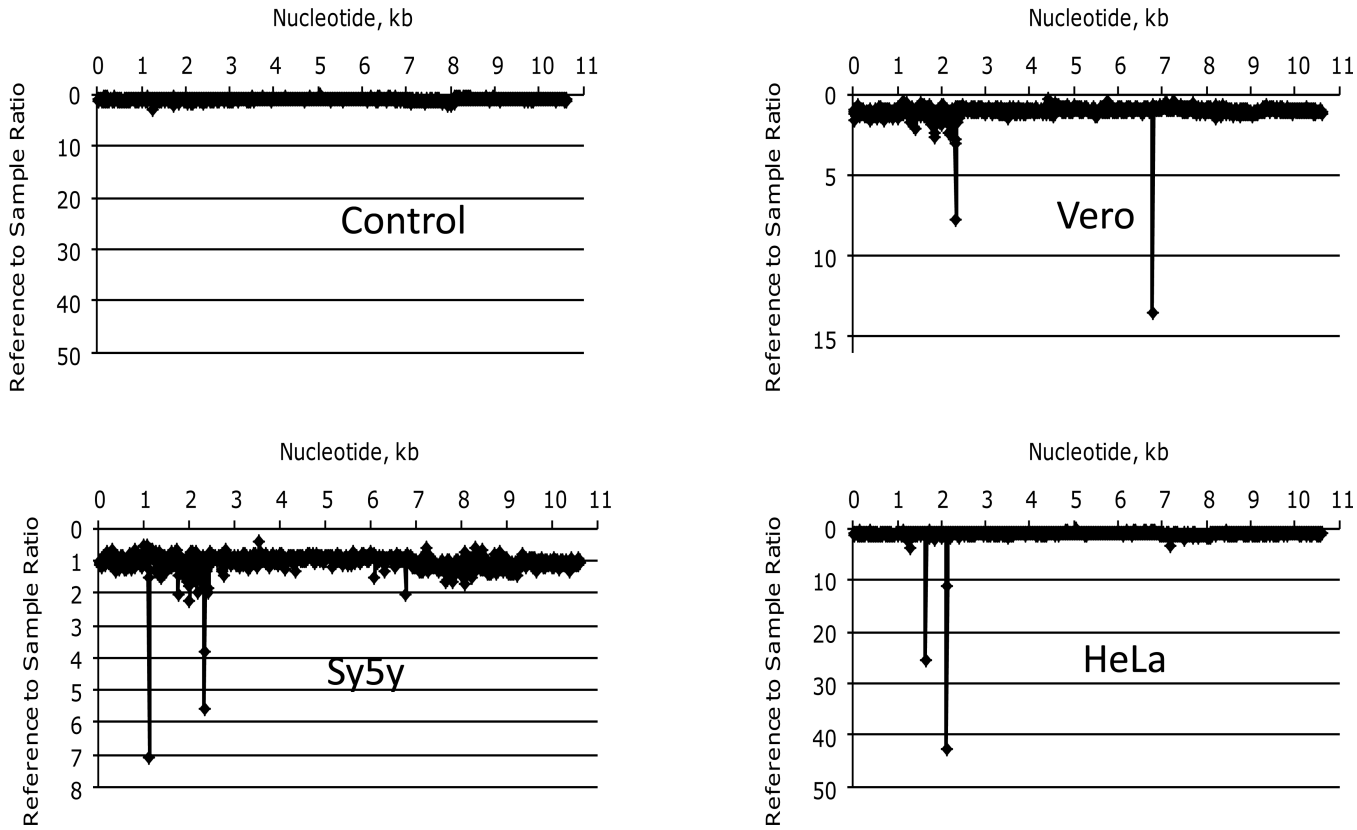

Figure 2.

Evaluation of genetic stability of the WN/DEN4Δ30 Lot 2 virus by the MARSH assay. The four panels show the ratios of hybridization signals of homogeneous reference DNA to cDNA from unpassaged virus (A), virus propagated 7 times in Vero cells (B), 6 times in SH-SY5Y human neuroblastoma cells (C) and 4 times in HeLa cells (D). The peaks on the ratio plots indicate the presence of mutations.

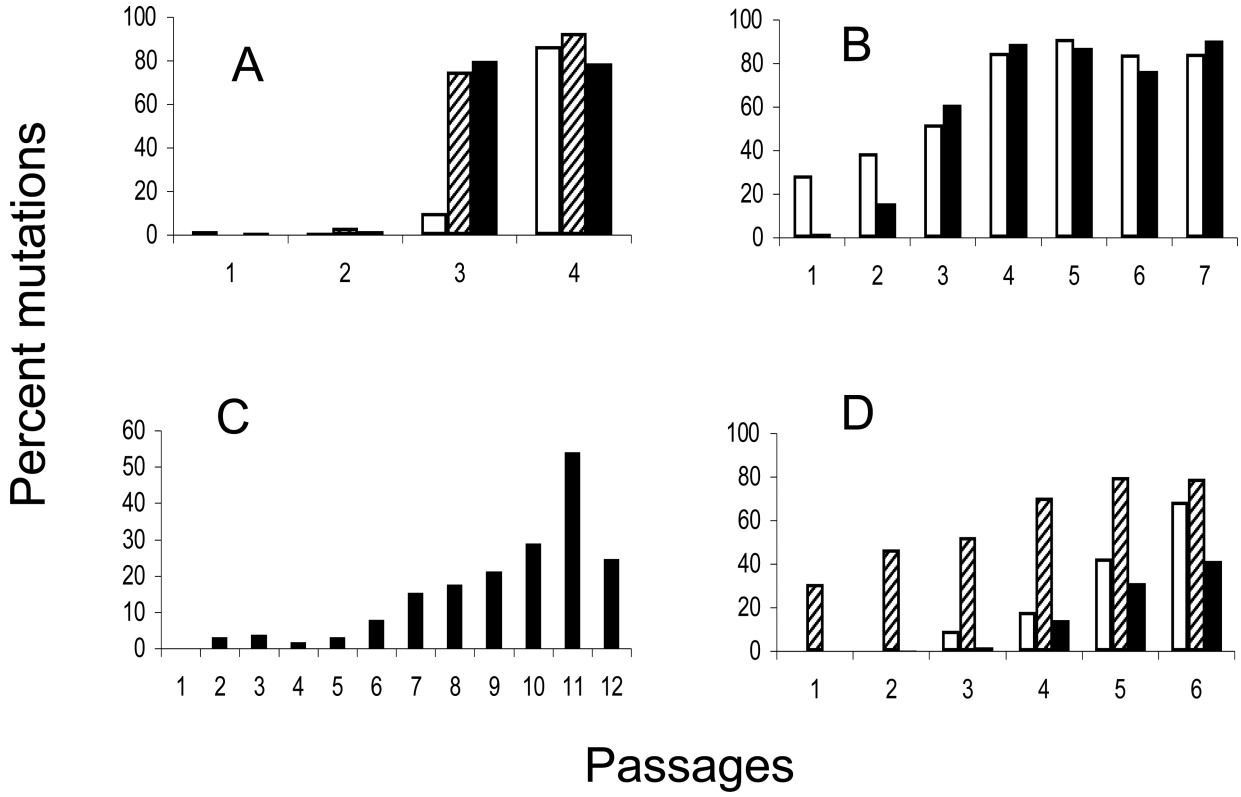

Figure 3.

Kinetics of accumulation of mutants in each passage of WN/DEN4Δ30 viruses propagated in cell cultures. **A**: Lot 2 virus passaged in HeLa cells; the nucleotide mutations were identified at positions 1657 (empty bars), 1285 (dashed bars), and 7153 (solid bars); **B**: Lot 2 virus passaged in Vero cells; the nucleotide mutations were identified at positions 2337 (empty bars) and 6751 (solid bars); **C**: Lot 1 virus passaged in Vero cells; a mutation at 8732 is shown; **D**: Lot 2 virus passaged in SH-SY5Y cells; the nucleotide mutations were identified at positions 2337 (dashed bars), 1111 (empty bars), and 6751 (solid bars).

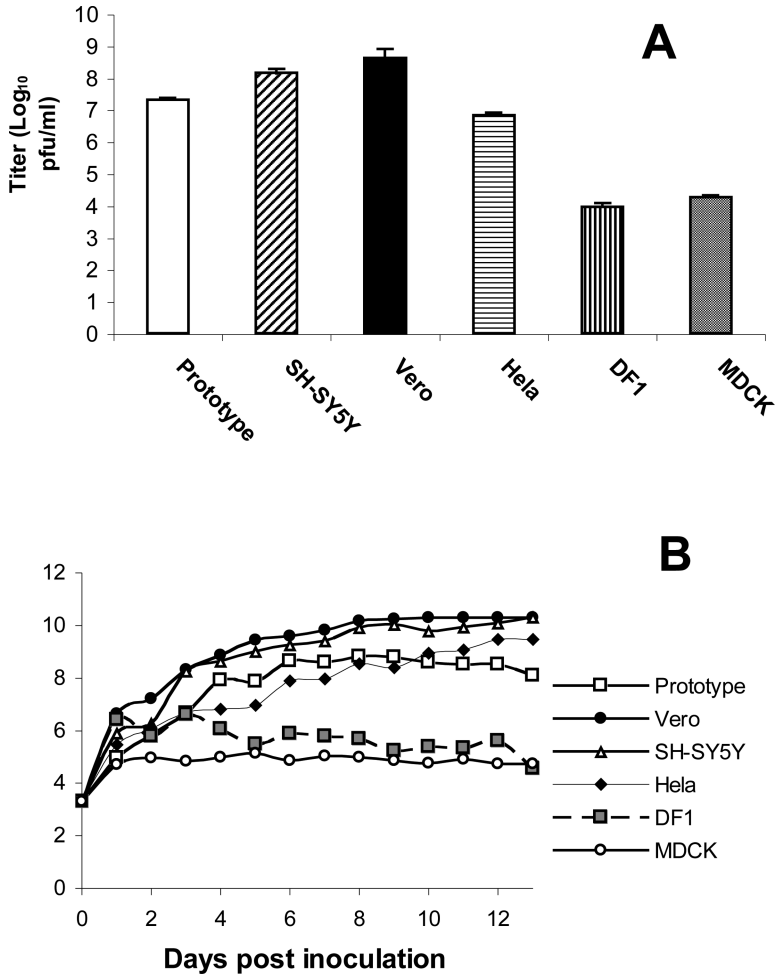

Figure 4.

Virus quantitation of Lot 2 stocks serially propagated 7 times in Vero, 4 times in HeLa, and 6 times in each of SH-SY5Y, MDCK and DF1 cells, and comparison with the unpassaged WN/DEN4Δ30 Lot 2 virus using a one-step quantitative RT-PCR assay. Panel A: Maximum virus titers determined by qRT-PCR in stocks of unpassaged and cell culture-passaged viruses. Panel B: kinetics of growth in Vero cells of parental WN/DEN4Δ30 Lot 2 virus (open squares) and stocks serially passaged in Vero (filled circles), SH-SY5Y (open triangles), HeLa (filled diamonds), DF1 (grey squares), and MDCK (open circles) cells. Vero cells were inoculated with parental or mutant virus at an MOI of 0.1, and the cell culture medium was harvested daily 13 days post-infection. Virus titer in cell cultures was determined by one-step quantitative RT-PCR. These results are presented as PFU/ml by using calibration curves obtained for viruses with known titers.

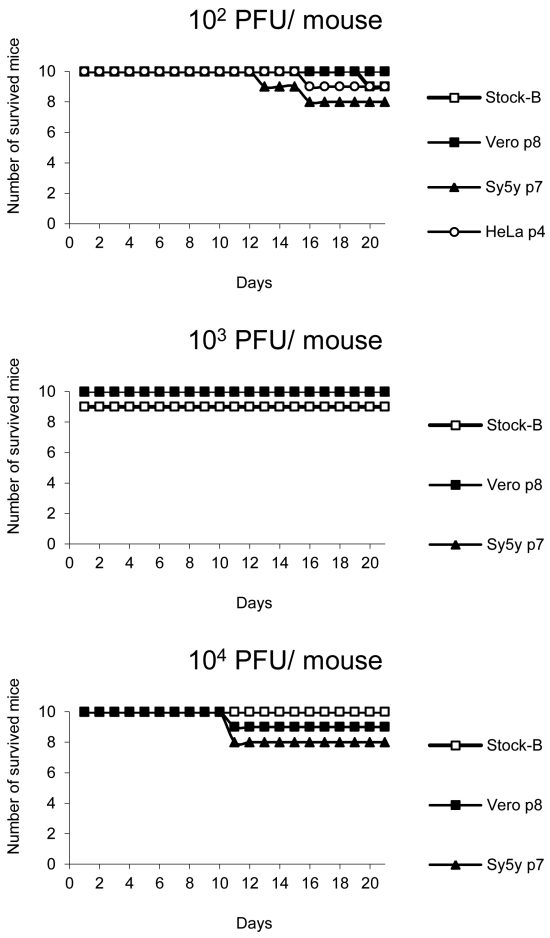

Figure 5.

Survival curves for 3-day-old Swiss Webster mice inoculated IC with parental WN/DEN4Δ30 Lot 2 virus (open squares) and stocks adapted to growth in Vero (closed squares), SH-SY5Y (triangles), and HeLa cells (circles). Comparison of morbidity of mice following inoculation with a dose of 10^2 (panel A), 10^3 (panel B), and 10^4 (panel C) PFU of virus. Mice were monitored for signs of encephalitis for 21 days and moribund mice were euthanized.

Table 1

Primers used to amplify specific regions from WN/dengue 4 chimera genomes, needed for preparation of micoarray fluorescent probes

Primer names	Primer sequences (5′ to 3′)	Tm (°C)	PCR amplicon positions	Sub-array numbers
WN1F	AGTTGTTAGTCTGTGTGGACCGACAAG	59	1-981	1
WN958RT7	ACGCGTTAATACGACTCACTATAGGCTCTGTTGCTCATTCCAAGGCAGT	73		
WN947F	CTATTGCTTTTGGTGGCCCCAGCT	60	924-2483	2
WN2460RT7	ACGCGTTAATACGACTCACTATAGGACTCCATGACGCCACACAACCCAT	72		
WN2421F	GGAATCACTCTGTTTCTGGGCTTC	56	2421-3543	3
WN3543RT7	ACGCGTTAATACGACTCACTATAGGCCATAGAAAAAGTTTCTGATGTGCCCG	72		
WN3477F	AAAGAAGAGAACATGGTCAAATCACAGG	58	3477-4581	4
WN4642RT7	ACGCGTTAATACGACTCACTATAGGAGGGGACGTCCCACAGGGCT	72		
WN4519F	TGACCTTATGGTACATGTGGCAAGTG	58	4519--6998	5
WN6998rT7	ACGCGTTAATACGACTCACTATAGGAGTCAGAATTGTGGTGGCTACTGCATAGAG	73		
WN6836F	CACCATCATTGGTCTAATAGC	49	6836-8635	6
WN8606RT7	ACGCGTTAATACGACTCACTATAGGGTTGTATCTGTCATGGCTAACTGAGTC	71		
WN8060F	AATAGAGGAAGGAAGAACATTAAG	51	8060-9617	7
WN9617RT7	ACGCGTTAATACGACTCACTATAGGCCTCTCATCTAGGGGCTTCACCA	73		
WN9211F	ACACAAGAATCACTGAGGATG	50	9211-10651	8
WN10651RT7	ACGCGTTAATACGACTCACTATAGGAGAACCTGTTGGATCAACAACACCAATCC	71		

Table 2

Primers and endonucleases used in MAPREC assay analysis

Mutation	Primer sequences 5′-->3′	Tm (°C)	PCR product size (bp)	Restriction Endonuclease
$A_{1272}T$	Sense: ATGACAAACGTGCTGACCCAGCTTTTGTGTGCAGAC	67	116	AseI
	*Antisense: GGCAAATTTGGCGCATGTGTCAATGCTTCCTTTGCCAATTA	67		
$T_{7186}C$	Sense: CCGCTCCACAGAATGGACCTCGGTGTG	66	140	Bpu10I
	*Antisense: GCTTTTGCCTGCAATCCTGGGCCTATTAT	62		
$T_{6784}C$	Sense: TTCTCATGGTACTGTTGATACCGGAACCAGGAA	63	118	BbsI
	*Antisense: ATCTCGTTGGCTGCTATTAGACCAATGATG	60		
$T_{8765}A$	Sense: CCAATTGGCTGTGGGCCCTCCTTGGAAAGAAGA**GTC**A	60	128	HincII
	*Antisense: TGTCCATCCCTGTTCTTCCTGAAAGACT	69		
$G_{2324}C$	Sense: CTTTGGATCAGTTGGAGGGGTGTTCACCT	63	147	SfaNI
	*Antisense: GAGTTGATTCCCATCCACAACAGAAGAGC	62		
$A_{1644}G$	Sense: ATGCGTACTACGTGATGACTGTTGGAACAAAG	66	128	Fnu4HI
	*Antisense: CTCCATTAACGTCTCTCTGTTCCTCCACGCAG	62		
$G_{1098}A$	Sense: TGCGTGACTATCATGTCTAAGGACAAGCCTACCAT	64	104	BbsI
	*Antisense: GTAGCCAAATAGCAATAACTGCGGACCTCTGCCAGGTTGGCCG**T**CT	73		

Note: The modified nucleotides are marked bold and undelined

*antisence primers were labeled with infrared IRDye-700 Dye

Table 3

Mutations detected following propagation of Stock B virus in cell cultures

Nucleotide change	Amino acid change	Gene	Vero p7	SY5Y p6	HeLa p4
G_{1098}àA	Glu_{49}àLys	E		+	
C_{1272}àT		E			+
A_{1644}àG	Thr_{225}àAla	E			+
G_{2121}àA	Glu_{490}àLys	E			+
G_{2324}àC	Met_{457}àlle	E	+	+	
A_{6784}àG	Lys_{125}àArg	NS4A	+	+/- *	
T_{7186}àC	Thr_{125}àPro	NS4B			+
T_{8765}àC	Asn_{390}àLys	NS5	+ †		

Note:

* mixture of mutant and non-mutant nucleotides

† mutation detected in passage 11 of Stock A virus