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# Inter- and Intra-Host Evolution of Dengue Viruses and the Inference to the Pathogenesis

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## 1. Introduction

Dengue viruses, like many RNA viruses are highly mutagenic, which have a potential to generate approximately one nucleotide mutation per round of genome replication (Domingo, Escarmis et al. 1996). The extent of genetic diversity differs among the sylvatic/urban cycles, chronologically isolates, serotypes and genotypes of Dengue viruses. All four serotypes of Dengue viruses (DENV-1 to DENV-4) evolved independently in their particular ecologic niche and could further classified into different genotypes among each serotypes based on nucleotide homology. Currently DENV-1 is classified into five different genotypes, DENV-2 into six different genotypes, DENV-3 into five different genotypes and DENV-4 into four different genotypes (Holmes and Twiddy 2003; Klungthong, Zhang et al. 2004; King, Chao et al. 2008; Vasilakis and Weaver 2008).

Genotype switch was correlated with DHF epidemic in certain region in recent years, which implied a more virulent strain may be evolved from the Southeast Asian and transmit to a new susceptible indigenous population to cause severe disease outcome (Thant, Morita et al. 1996; Sittisombut, Sistayanarain et al. 1997; Cologna and Rico-Hesse 2003). Our previous studies showed that the larger of quasispecies among structure proteins than non-structural proteins, which probably implied the more selection immune pressure on the structure proteins instead of merely randomly changing during replication (Chao, King et al. 2005). This has been further validated by the data in E gene where the Domain I, the antigenic sites had larger sequences diversity than other domains. An increasing question raised upon this observation has focused on the concept that whether quasispecies distribution of dengue viruses as a reservoir of virus variants plays an essential role in diversification and selection of variants which replicates better in human population in the context of the high incidence density and clustering of dengue epidemic. Therefore, to closely examine the inter- and intra-host evolution of dengue viruses among full-genomic sequence will be required to elucidate the relationship between host immune responses, viral strain infected and disease outcomes.

The recent epidemics of dengue in Taiwan started when dengue virus serotype 2 (DENV-2) was first introduced into the southern off-islet of Hsiao-Liu-Chiu in 1981 after its absence for 38 years since World War II (WWII) (Tsishe 1932; Wu 1986). Later in 1987-1988, the epidemic

of DF, caused by DENV-1 mainly in Kaohsiung and Pingtung in southern Taiwan. Tainan City, located just at north of Kaohsiung, had not had a dengue epidemic since l942-43 until three outbreaks occurred recently (Ko 1989; Chen, King et al. 1997). The first of three DF epidemics in Tainan, was caused by DENV-1, resulted in 38 confirmed DF cases in 1994 with no DHF cases observed. Three years later in 1997, a more localized DENV-2 outbreak of DF occurred, involving only 14 confirmed cases. The third epidemic of dengue, attributed to DENV-3, began in late 1998 and continued into January, 1999. During this 1998-1999 epidemic, 142 confirmed dengue cases including at least 14 DHF cases were officially reported (Chao, Lin et al. 2004).

During the epidemic, two interesting epidemiological phenomena were found based on our previous publications(Chao, Lin et al. 2004). First, the DHF/DF ratio increased with time, from 11% during the first time interval, to 20% and 30% during the second and third time intervals, respectively. Second, the majority (73.3%, 88/120) of the dengue cases were primary infections, including DHF, which showed no significant association with secondary infections [13 DHF cases had primary infection and 8 DHF cases had secondary infection, odds ratio=1.92 (95%CI 0.64-5.76), p=0.19]. Therefore, we hypothesize that intense transmission of dengue virus within closed environment may drive emergence of DENV-3 strain with higher propensity of causing sever disease. Thus, viruses, isolated in first passaged-C6/36 cell culture, from three well-characterized family clusters were chosen for molecular genetic study.

Since previous study suggested that there were a genetic marker in DENV-2 viruses, which differentiated American genotype with the southeast Asian genotype and caused wind-sweeping epidemic in the central and south America since 1981 epidemic in Cuba(Leitmeyer, Vaughn et al. 1999; Sariol, Pelegrino et al. 1999; Rodriguez, Alvarez et al. 2005; Rodriguez, Alvarez et al. 2005), it will be interesting to look at the DENV-3 viruses whether such genetic marker or virulence marker exists or not. We started by the consensus-direct sequencing of full-length genome to identify the genetic markers associated with virulence and the most probable quasispecies regions. Candidate regions were then amplified, cloned, and randomly selected multiple clones were sequenced to better understand the population dynamic of quasispecies variation among family pairs. The clonal sequencing result was also conducted in selected regions by obtaining amplicons directly from viremic plasma and *Aedes aegytie* passage one virus.

## 2. Materials and methods

### 2.1 Case definition

A confirmed dengue case was defined as a person with the illness that fulfilled any of the following laboratory diagnostic criteria: (1) isolation of dengue virus from serum; or (2) identification of dengue-specific cDNA fragment from plasma or serum by reverese-transcriptase polymerase chain reaction (RT-PCR)(Lanciotti, Calisher et al. 1992); or (3) seroconversion of dengue-specific IgM from negative to positive but seronegative for Japanese encephalitis (JE)-specific IgM by IgM-enzyme-linked immuno-sorbent assay (IgM-ELISA)(Shu, Chen et al. 2003); or (4) a 4-fold or greater titer rise in dengue-specific IgG antibody in paired serum samples(Shu, Chen et al. 2002). The clinical diagnosis of DHF was based upon revised WHO's criteria in 1997(World Health Organization 1997), as follows: (1)

fever, (2) hemorrhagic manifestations, including a positive tourniquet test result, (3) thrombocytopenia (100,000/$mm^3$ or less), and (4) evidence of plasma leakage manifested by at least one of the following: hemoconcentration, presence of pleural effusion or ascites (documented by radiography, ultrasound, or computed tomographic scan) or hypoproteinaemia. Hemoconcentration, which was defined as a 20% increase in hematocrit compared with stabilized hematocrit at hospital discharge or revisit after discharge, was calculated as the ratio of the difference of maximum and minimal hematocrit values, divided by the minimal value. In consideration of references used in most hospitals in Taiwan, hypoproteinaemia was defined as a serum albumin level less than 3 gm/dL. Those confirmed dengue cases were classified as primary, secondary, or indeterminate infections, depending on the ratio of DENVVgue-specific IgM/IgG as measured by the capture IgM and IgG ELISA test(Vaughn, Nisalak et al. 1999; Shu, Chen et al. 2003).

### 2.2 Family cluster chosen

In choosing family clusters during 1998 epidemics, minimum of one confirmed dengue patient within the same household unit were selected and it ended up with 12 family clusters identified. The definite disease classification, disease onset date, and detail demographic data were recorded for all dengue patients in the same family clusters. Virus isolates for molecular genetic characterizations from three family clusters were selected, based on the following criteria: (1) with DF and DHF patients in the same family clusters; (2) the duration of disease onset between DF and DHF patients in the same family clusters is longer than 3 day and shorter than 10 days. This is based on the assumption of that if the dengue virus is transmitted within the same household from DF patient to DHF patient and the duration of disease onset between two cases as indicated, there is a high propensity that virus may transmit by mosquito mechanically, influenced by the multiple feeding behavior of *Aedes aegytie*. The bottleneck transmission by the mechanical transmission may create the opportunity of transmitting the higher virulent virus population from DF patient to naïve individual who may have higher odds ratio of developing DHF. The detail description of basic information including onset date, sex, age, viral load and immune status for three family clusters was summarized in Table 1.

| Cluster | ID | Disease status | Onset date | Age | Sex | Immune status | Viral load (RNA copies/ml plasma) |
|---|---|---|---|---|---|---|---|
| 1 | 364 | DF | 11/20 | 38 | F | Primary | 107,000 |
| 1 | 368 | DHF | 11/27 | 27 | M | Primary | 3,890,000 |
| 2 | 390 | DF | 12/1 | 69 | M | Secondary | <600 |
| 2 | 388 | DHF | 12/5 | 57 | F | Primary | 1,540,000 |
| 3 | 414 | DF | 12/7 | 36 | F | Primary | <600 |
| 3 | 407 | DHF | 12/12 | 63 | M | Secondary | 1,360,000 |

Table 1. The Demographic Data and Viral Load From Each Patient Within the Family Clusters.

### 2.3 Virus isolation

Acute-phase serum or plasma samples were collected from patients within seven days after the onset of fever and stored in –70°C freezer until tested. Plasma sample aliquots were used

to infect C6/36 *Aedes albopictus* mosquito cell lines as described previously(Kuno, Gubler et al. 1985) and were identified as DENVV-3 by indirect fluorescent antibody tests with serotype-specific monoclonal antibodies (DENV-1:H47, DENV-2:H46, DENV-3:H49, DENV-4:H48). Briefly, C6/36 cells were seeded into 75-$cm^2$ tissue culture flasks at $5x10^5$ cells per flask in Mitsuhashi & Maramorosch insect medium (MM) (Sigma, St. Louis, MO) and Dulbecco's minimum essential medium (DMEM) (Invitrogen, San Diego, CA) with 1:1 ratio containing 10% fetal calf serum and 100% antibiotics-antimycotics (Invitrogen, San Diego, CA). As cells reached 80% confluent after seeding, the medium was removed and only 1ml left. 40 ul of patient's plasma was added to each flask and the virus was allowed to infect cells by rolling the flasks every 15 minutes for 2 hours. After absorption, fresh maintenance medium containing only 2% fetal calf serum was added and incubated in 37°C incubator. The culture supernatant was collected at day 7 and 14 post infection, and used to re-infect BHK-21 cells for plaque assay to determine virus titer as previously described. For the molecular sequencing, the C6/36 cell one-passaged virus was used for full-length consensus sequencing to identify potential heterogeneous regions for clonal sequencing as described later.

### 2.4 Preparation of viral RNA and RT-PCR amplification

Dengue viral RNA was isolated either from viremic plasma or C6/36 passaged one supernatant by using QIAamp viral RNA mini kit (Qiagen, Germany) following the manufacturer's protocol. The eluted RNA was subjected to Titan™ one tube RT-PCR System (Boehringer Mannheim) to amplify overlapping regions of DENV-3 sequence by virus specific synthetic oligonucleotide primers. The oligonucleotide primer pairs were designed based on published full-length DENV-3 sequence data for H87 and 80-2 obtained from Genebank at the National Center for Biotechnology Information (NCBI) and some unpublished DENV-3 sequences (personal communication, Chang et. al.; Centers for Disease Control and Prevention, Fort Collins, CO., U. S. A.). Ten overlapping fragments were generated which spanned genomic regions 1 to 1181, 530 to 1694, 1259 to 1694, 2171 to 3417, 3142 to 4677, 4123 to 5686, 5443 to 7477, 7246 to 8750, 8501 to 10316, 9991 to 10688 as previously described(Chao, King et al. 2005).

### 2.5 PCR product cloning, purification and sequencing of PCR fragments

PCR product was purified by using the QIAquick PCR Purification Kit Protocol following manufacture suggested protocol (Qiagen, Germany). The purified double-stranded DNA fragments were subjected to sequence analysis the cycle-sequencing dye terminator method using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Applied Biosystems, Foster City, Ca.). We estimated the amount of DNA for each sequence reaction by comparing the band intensity of a 1:10 diluted product (1 μL product + 9μL DEPC dH20) with 2 and 4μL of the high DNA mass ladder (Invitrogen, San Diego, CA) after gel electrophoresis using EtBr containing 1% agarose gel. For each sequencing reaction, approximately 50 to 100 ng purified DNA was combined with 3.2 pmol of sequencing primer (3.2 μL of 10uM primer concentration), 8.0 μL of reaction cocktail (containing dNTP, dye-labeled-ddNTP terminators and Taq polymerase) and deionized distill water to bring the final volume to 20ul. Start on sequencing cycling program on the thermocycler as suggested in the manufacturer's protocol (30 cycles of 96°for 10sec; 50°C for 5sec; 60°C for 4 min) and hold at 4°C forever.

The reaction mixture was column purified by home-made Sephadex™ G50, fine DNA grade (Amersham Pharmacia, Biotech, AB, Sweden) filled column, and the DNA was dried in a vacuum centrifuge for 20 minutes. Finally, the DNA pellet was resuspended in Hi-D formamide (denaturing reagent), transferred to a 96-well plate, heated for 2 min at 95°C and kept on ice prior to run the 3100 automate sequencer (Perkin-Elmer, Aplied Biosystems). We used a short capillary (47 cm by 50 um diameter) and Performance Optimized Polymer 6 for the run.

The PCR product from the potential heterogeneous regions ligased with the T/A cloning vector, PCRII-TOPO, was used to transform to *E. coli* TOP10 competent cells (Invitrogen, San Diego, CA). At least 30 recombinant clones were randomly selected, and completely sequenced by using insert flanking primers, T7 and cSP6.

### 2.6 Mosquito feeding and inoculation

To determine the sequence diversity inside the mosquitoes, 70 5-day old adult female mosquitoes of each *Ae. aegypti* and *Ae. albopictus* were starving for 2 days prior to oral feed on virus spiked rabbit blood. Mosquitoes of each species were enclosed in two fine mesh net covered cans. A mixture of 5 ml fresh-prepared rabbit blood (with heparin), 1 ml 1% sucrose and 2 ml virus stock ($10^6$PFU/ml) was spreading over the net at room temperature for one hour for mosquito to feed on virus-spiked blood mixture. One ml each of the blood mixture was collected before feeding, feeding 0.5 hour and after feeding, centrifuged at 3000rpm for 10 minutes to collect the supernatants. The virus titer in each collection was determined by the plaque assay. Mosquitoe pools were relocated to the insectory room for 14 days after blood feeding. Ten other female *Ae. aegypti* mosquitoes were inoculated with the 1:5 dilution of viral stock by intrathoracic inoculation techniques.

Virus infected mosquitoes were hold at 32°C for 7 days and 14 days at which the salivary glands were dissected from surviving females, and the presence or absence of viral antigen in these tissues was determined by the indirect fluorescent antibody techniques (IFA). The salivary glands from oral feeding or intrathoracically inoculated mosquitoes were placed inside the 1.5ml eppendorf with 200ul PBS, and frozen at –70°C before RNA extraction.

### 2.7 Nucleotide and Amino acid sequence analysis

Overlapping chromatogram files retrieved from the automate sequencer were analyzed and edited using the SeqMan program in the Lasergene software package (DNASTAR, inc. Madison, Wis.). The derived consensus sequences after excluding the sequences of primers were aligned using GCG package (Genetic Computer Group, Wis.). For full-length genomic sequences we paid special attention for the regions consistently presented mixed-chromatographic picks. These regions were identified and selected for the clonal sequence analysis. Pairwise comparisons of both nucleotide and amino acid sequences between isolates and clonal sequences were performed using the program MEGA v2.1 (Molecular Evolutionary Genetics Analysis, Pennsylvania State University, PA) to determine the mean and range of proportion of difference (hamming and p distance)(Kumar, Tamura et al. 2004). The obtained nucleotide sequences were aligned with the sequences of available DENV-3 strains and the DENV-2 Jamaica strain obtained from the Genebank at NCBI, using the multiple sequences alignment program PILEUP with the default gap penalties. The

PHYLIP package, that utilized the neighbor-joining method to calculate nucleotide evolutionary distances, was used to generate a phylogenetic tree.(Felsenstein 1993)

### 2.8 Statistical analysis

All the data from the questionnaires and laboratory results were entered into the database and analyzed by SAS (Statistical Analytical System, Wisconsin, 6.12 version). Chi-square test was performed to compare differences in two groups for discrete data. The exact p-values by Fisher exact test were calculated if the expected number was smaller than 5.

## 3. Results

### 3.1 Geographical distribution and chronological spread of genotypes

DENV-3 was the only serotype isolated during the 1998 epidemic, which was identified in 38 (26.7%) of the confirmed dengue cases. The phylogenetic tree analysis showed that the virus strain belongs to DENV-3 genotype 2, which comprises Thailand strains according to the classification of Lanciotti et al (Fig 1)(Lanciotti, Lewis et al. 1994). Further examining the phylogenetic analysis results based on full-length genomes by neighbor-joining method for DENV-3 viruses including 4 different genotypes and other serotypes of dengue viruses suggested the similar results with previous reports by Wittke et al(Wittke, Robb et al. 2002), which separated the viruses into 4 main groups except genotype 4 because of lacking full-length sequence. The proximity of different genotypes of DENV-3 was that genotype I and IV were closer than genotype type II and III. After comparing the full-length sequences of the DF and DHF isolates, no genetic makers can be found to differentiate the disease severity, which might suggest the genomic virulence determination site does not exist.

Next, we constructed a contingency table to analyze all strains by geographical regions and genotypes (Table 2). Based on the geographical distribution of isolates by time, the Philippines-Indonesia-Malaysia region has all genotypes except traditional American genotype (genotype IV). The oldest genotype V represented the DENV-3 prototype H87, which rarely being isolated except one in China in 1980 and the other in Malaysia in 1981. It is very possible genotype I evolved from genotype V and first appeared also in the Philippines-Indonesia-Malaysia region since 1974 in Malaysia and 1973 in Indonesia. It later spread into other southeast Asian countries, including Taiwan and further into south Pacific Island, including Fiji in 1992 and Tahiti in 1989. Genotype II however, first evolved from Thailand in 1962 and later spread into Malaysia and replaced the original genotype I to cause epidemic locally in 1993-4. Genotype III was the wide-spread genotype, which evolved probably also from the Philippines-Indonesia-Malaysia region and in the 1980's this genotype spread west to India, Sri Lanka and Africa and east to Taiwan. Genotype III was the only genotype isolated in the Latin America since 1994, which the introduced Asian genotype replaced the American genotype caused a great DF/DHF epidemics in many countries. Genotype IV was the traditional American genotype rarely isolated in other countries. Its distribution was limited in Puerto Rico in 1963 and 1977 and in Tahiti in 1965, which suggested virus exchange between west-Pacific region and the Central- America region during 1960s.

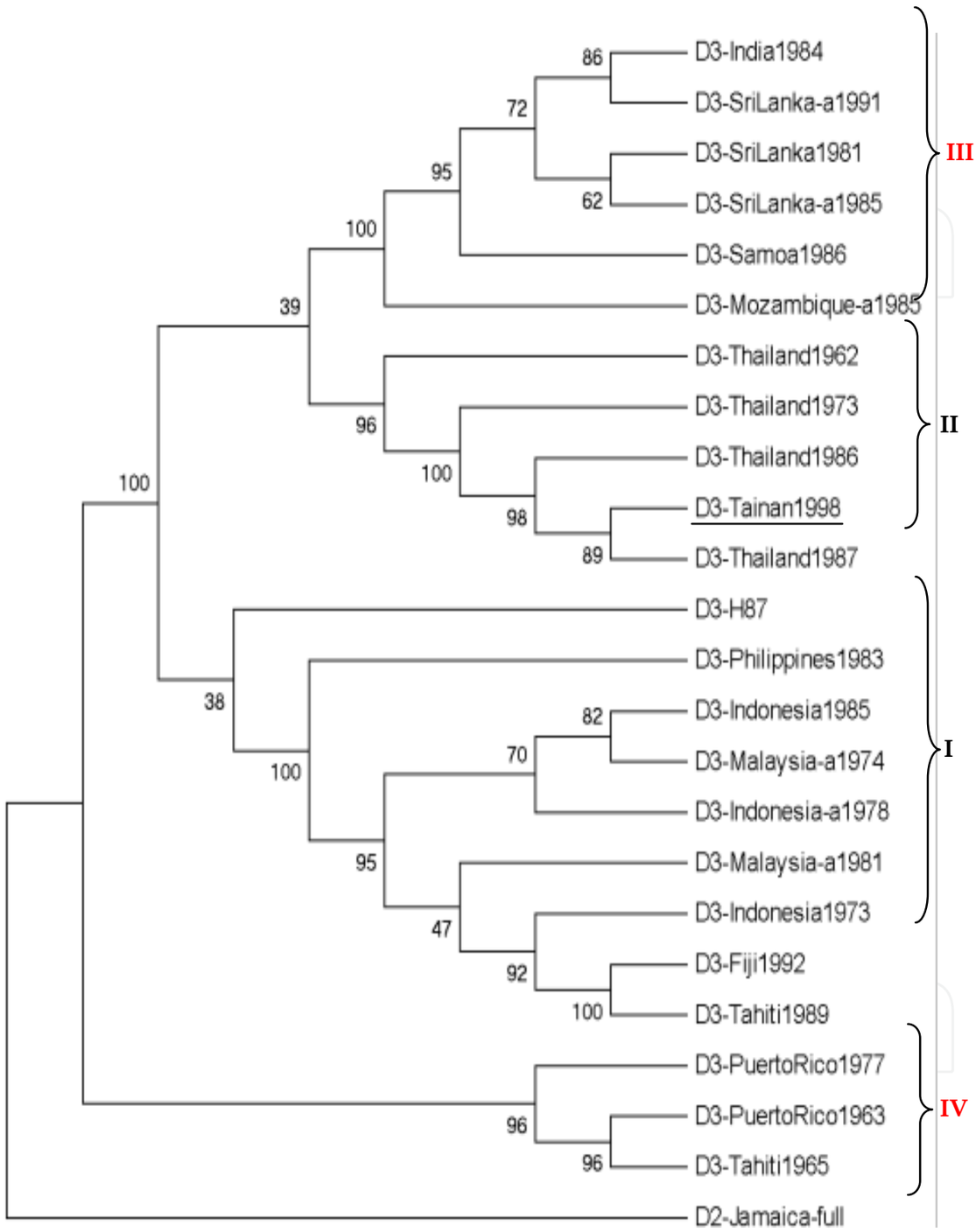



Fig. 1. Phylogram Generated by Parsimony Analysis of Nucleic Acid Sequences from PrM/M and E Genes of 24 DENV-3 Viruses Isolates.The numbers displayed above the horizontal lines correspond to the percentage of bootstrap analysis. Parsimony analysis was performed by the heuristic branch swapping alogorithm of MEGA-2.

| Geographical region | Country | Genotypes | | | | |
|---|---|---|---|---|---|---|
| | | I | II | III | IV | V |
| Philippines-Indonesia-Malaysia region | Philippines | 1983 | | | | 1956 |
| | Malaysia | 1974, 1981,1997 | 1992,1993,1994 | | | 1981 |
| | Indonesia | 1973,19788, 1985,1989 | 1976 | | | |
| Thailand-Vietnam | Vietnam | | 1994,1996 | | | |
| | Thailand | 1988 | 1973,1986-1998 | (1962) | | |
| | China | | | | | 1980 |
| Pacific islands | Polynesia | | | | 1964,1969 | |
| | Tahiti | 1989 | | | 1965 | |
| | Fiji | 1992 | | | | |
| south Asia | Sri Lanka | | | 1981,1985, 1989,1991 | | |
| | India | | | 1984 | | |
| Africa | Somoa | | | 1986 | | |
| | Mozambique | | | 1985 | | |
| Central/south America | Guatemala | | | 1996-98 | 1977 | |
| | Mexico | | | 1995-97 | | |
| | Puerto Rico | | | | 1963 | |
| | Brazil | | | 2000 | | |

Table 2. Geographical Distribution of DENV-3 Genotypes.

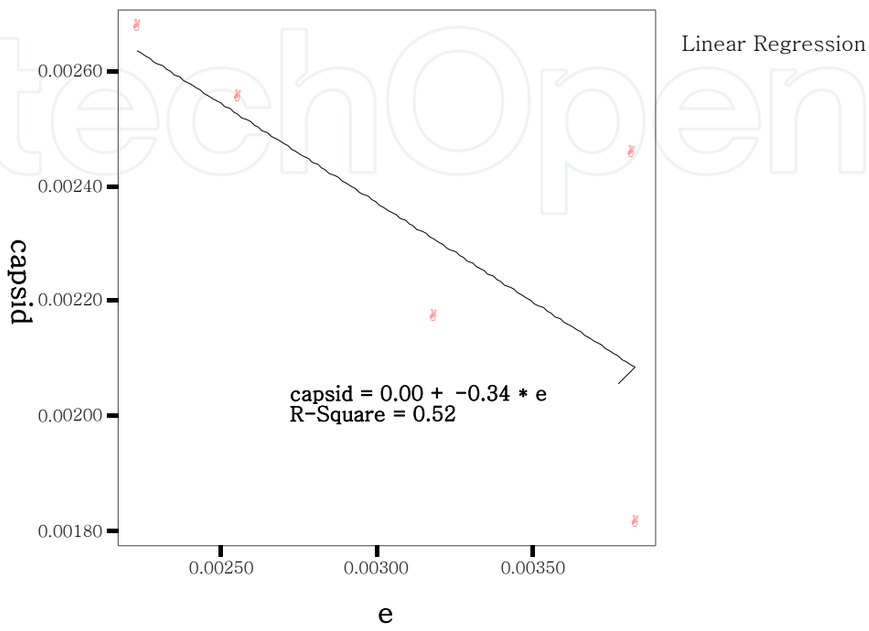
### 3.2 Nucleotide and amino acid sequence diversity among different regions of full-genome

If DHF patients were not caused by secondary infection or more virulent strain of DENV-3 viruses during 1998 epidemic, would it be possible that there existed a sub-variant in the quasispecies of DENV-3 viruses which caused more severe form of disease after dengue viral infection? Three family clusters were chosen as states in Material and Methods section. More than 20 clones containing the PCR products of different genes of dengue viruses from 6 dengue patients were completed sequenced, aligned and analyzed by excluding the primer sequences. To examine the extent of sequence variation, we determined the mean pairwise p-distance, which is the number of substitution divided by total nucleotide (amino acids) sequenced for each pair of clones. The results were summarized in Table 3. In genearal, non-structural protein such as NS3 and NS5 had the least sequence diversity than structural protein such as capsid or envelope protein. Among non-structural protein, NS3 also presented the least sequence diversity with mean p-distance of nucleotide ranged from 0.09-0.24% and that of amino acid ranged from 0.2-0.5%. Among structural protein, envelope protein presented the largest sequence diversity with mean p-distance of nucleotide ranged from 0.2-0.4% and that of amino acid ranged from 0.4-0.8%. The difference of mean pairwise p-distance among different genes was statistically significant ($p<0.01$).

| ID | C-PrM | | | E | | | NS3 | | | NS5 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | No of clones | nucleo-tide | Amino acid | No of clones | nucleo-tide | Amino acid | No of clones | nucleo-tide | Amino acid | No of clones | nucleo-tide | Amino acid |
| 364 | 23 | 0.002671 | 0.006671 | 26 | 0.00223 | 0.004316 | 19 | 0.000936 | 0.003287 | 13 | 0.00174 | 0.004133 |
| 368 | 29 | 0.00245 | 0.004863 | 13 | 0.003815 | 0.006638 | 27 | 0.001182 | 0.003053 | 17 | 0.002416 | 0.00348 |
| 388 | 21 | 0.002027 | 0.004001 | 25 | 0.003878 | 0.006436 | 26 | 0.001247 | 0.00323 | 18 | 0.003005 | 0.006095 |
| 390 | 22 | 0.002165 | 0.004183 | 13 | 0.003179 | 0.00761 | 25 | 0.002437 | 0.005662 | 25 | 0.002459 | 0.003603 |
| 407 | 13 | 0.001807 | 0.004416 | 23 | 0.003828 | 0.006235 | 23 | 0.001592 | 0.001968 | 16 | 0.004251 | 0.006818 |
| 414 | 24 | 0.002549 | 0.004901 | 20 | 0.002555 | 0.004773 | 18 | 0.001013 | 0.001639 | 26 | 0.003697 | 0.006487 |
| Mean | 22 | 0.002278 | 0.004839 | 20 | 0.003697 | 0.006487 | 23 | 0.001401 | 0.00314 | 19 | 0.003122 | 0.005915 |

Table 3. Sequence Diversity (Mean p-Distance) Among Different Genome Regions of dengue Viruses.

We next examined the relationship between the extents of sequence variation among different genes. As shown in Fig. 2, there was a trend of increase in the mean pairwise p-distance of nucleotides of envelope protein as that of the NS3 protein increase (simple linear regression, $r=0.6$, $p=0.01$). Similarly, a linear relationship was also observed when comparing the mean pairwise p-distance of amino acid between these two genes (Fig. 3).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0.00220
0.00200
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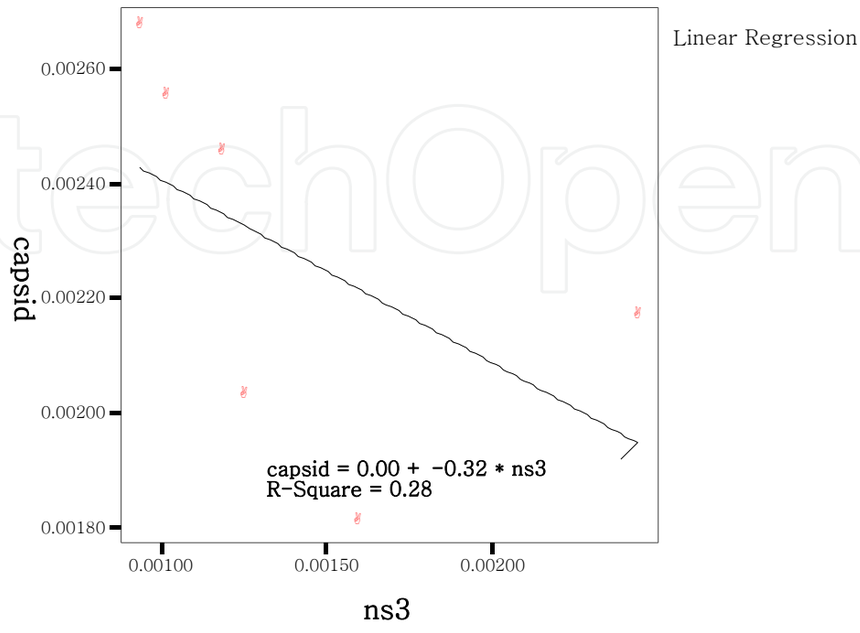
R-Square = 0.52
0.00250
0.00300
0.00350
e

**(a)**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0.00200
ns3

**(b)**

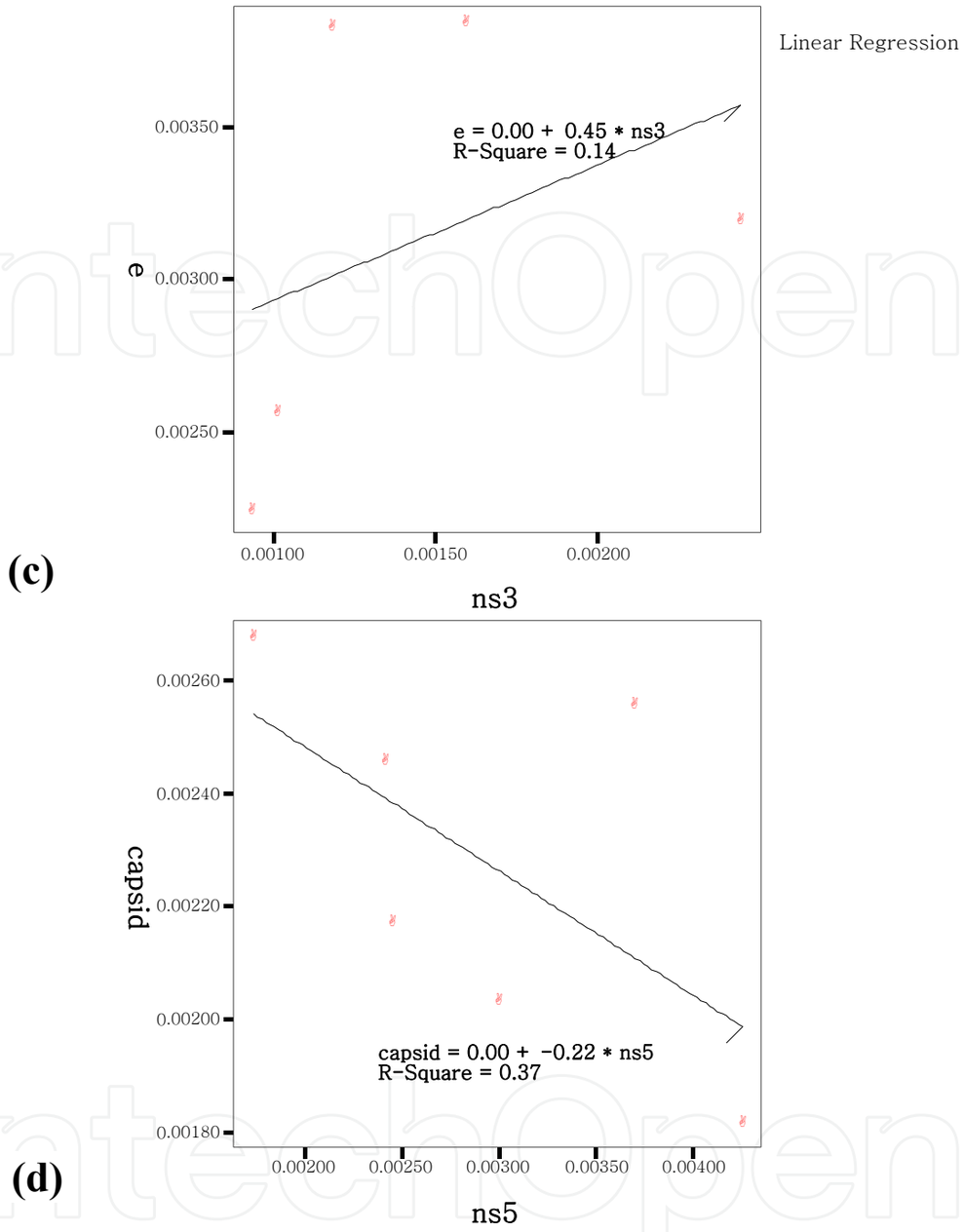
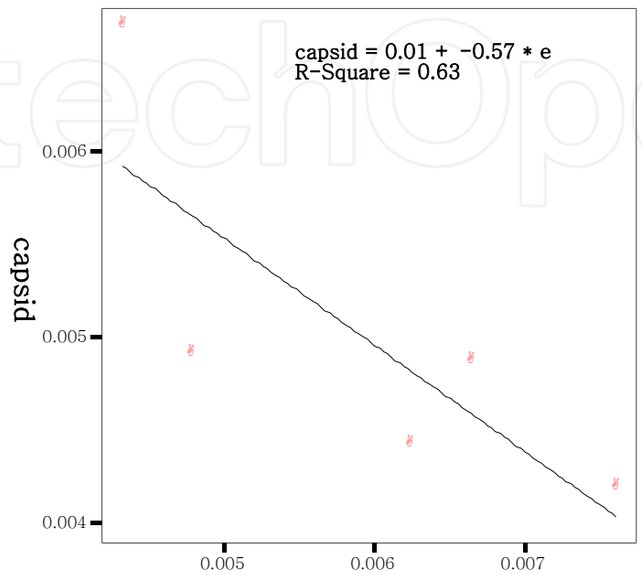



Fig. 2. The Relationship of Intrahost Nucleotide Sequence Diversity Between Different Genomes. Y-axis and X-axis represented the mean p-distance of nucleotides of indicated protein from different patients' isolates. The line was fitted regression line generated by SPSS software and the upward line indicated the positive correlation; the downward line indicated the negative correlation. (a) showed the relationship of mean p-distance between capsid and envelope protein; (b) showed the relationship between capsid and NS3 protein; (c) showed the relationship between NS3 and envelope protein; (d) showed the relationship between capsid and NS5 protein. Only the regression line in (c) showed statistical significance, meaning the higher sequence diversity in envelope protein correlated with the higher sequence diversity in NS3 protein.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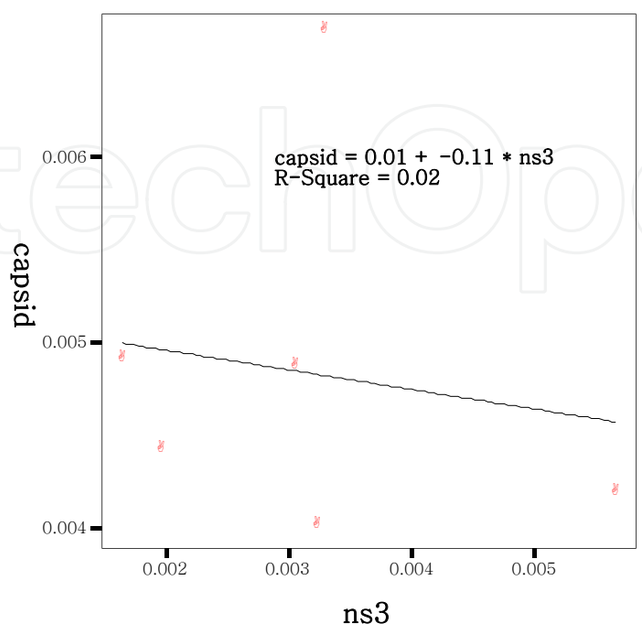
0.005
0.006
0.007
e

**(a)**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ns3

**(b)**

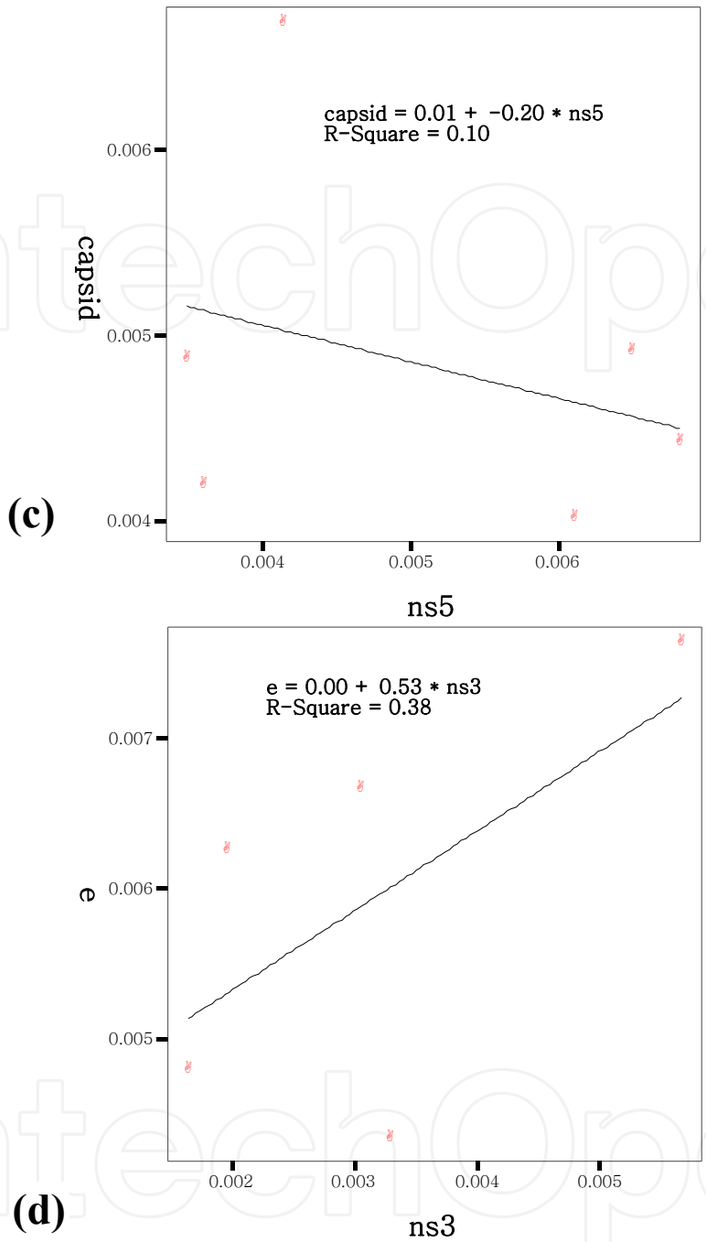


Fig. 3. The Relationship of Intrahost Amino Acid Sequence Diversity Between Different Genomes. Y-axis and X-axis represented the mean p-distance of amino acid of indicated protein from different patients' isolates. The line was fitted regression line generated by SPSS software and the upward line indicated the positive correlation; the downward line indicated the negative correlation. (a) showed the relationship of mean p-distance between capsid and envelope protein; (b) showed the relationship between capsid and NS3 protein; (c) showed the relationship between NS3 and envelope protein; (d) showed the relationship between capsid and NS5 protein. Only the regression line in (d) showed statistical significance, meaning the higher sequence diversity in envelope protein correlated with the higher sequence diversity in NS3 protein.

### 3.3 Correlation between sequence diversity and phenotypic change

The relationship between genotype and phenotype has been an interest among evolutionary biologists and virologist(Clarke, Duarte et al. 1993; Bielefeldr-Ohmann and Barclay 1998; Arias, Lazaro et al. 2001). Several examples of viral clones isolated from mutant spectra showed altered biological properties, such as HIV mutants with resistant to antiviral inhibitors(Farci, Shimoda et al. 2000; Delwart, Magierowska et al. 2002). The correlation between quasispecies mutant clones with DHF phenotypic change has not been discussed before. With extensive searching, there was no such genetic marker of subvariants from clonal sequencing were found among those family clusters. However, by comparing intra-host variation, there was concomitant increase sequence diversity along with the decrease of identical clones from the first case to the other one in the same family cluster. As shown in Fig. 4, three family clusters present consistent increase sequence diversity from 2.3, 2.5, 3.0 in first case in each family cluster to 4.8, 4.0, 3.3 in the second case in each family cluster.

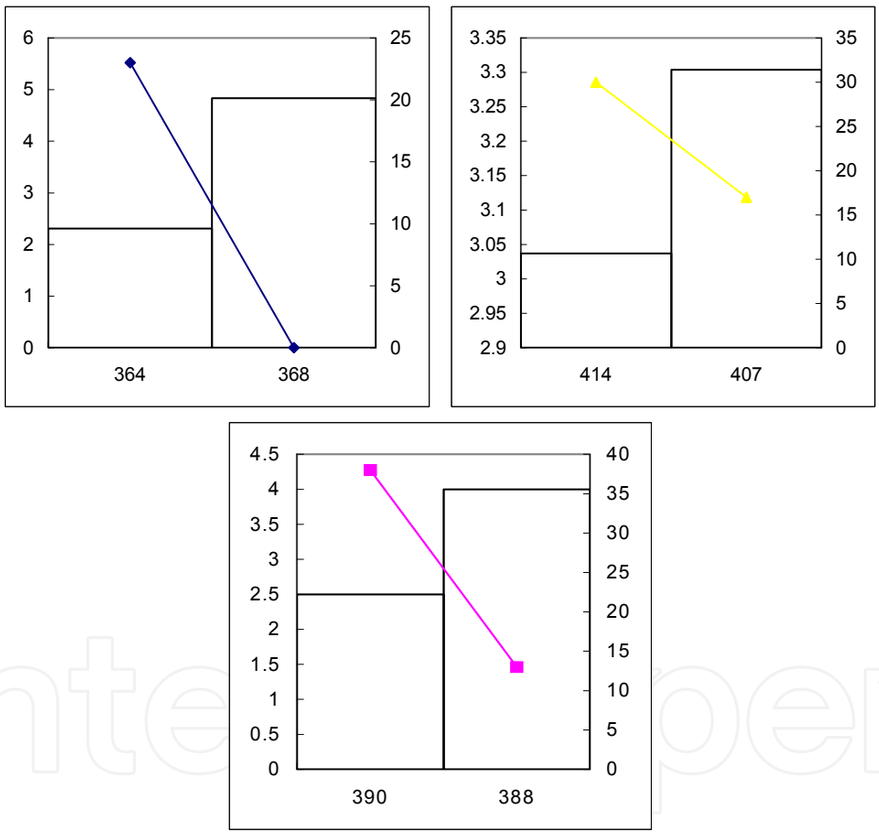


Fig. 4. Genetic Distance Among the Variants and Percent of Identical Clones Between Patients With Different Disease Manifestations Within Individual Family Cluster. Y-Axis on the left indicated the mean hamming distance and on the right indicated the percentage of identical clones, measured by calculating the number of identical clones among total clones sequenced. The box represented the value of mean hamming distance of indicated patient ID shown on the bottom and the line represented the value of identical clones percentage of indicated patient ID shown on the bottom. (a)-(c) indicated the three family clusters with DF and DHF in the same family cluster. The increase of mean hamming distance correlated with the decrease numbers of identical clones.

Consistent with sequence diversity, the percentage of identical clones decreased from 23%, 38%, 30% in first case in each family cluster to 0%, 13%, 17% in the second case in each family cluster. This kind of consistency was correlated with viral load, but not with primary or secondary infection, sampling date, age or sex.

### 3.4 Comparison of sequence diversity among original plasma, cultured viruses and mosquito inoculation

Little is known about the sequence diversity within mosquitoes, original plasma of patients and cell line passaged viral stocks. The sequence diversity among three family clusters was reconfirmed in the patient's original plasma and mosquito inoculation by one passage viral stock. 30 clones were picked after PCR direct cloning and analyzed after direct sequencing. There was also consistent change with the extent of quasispecies and the number of identical clones. It was found that the sequence diversity of nucleotide (hamming distance) was the lowest in original plasma (1.4) than that in one passage viral stock (2.5) or mosquito inoculation (1.7) at patient ID 368. Although we did not do mosquito inoculation for patient sample ID 388 and 407, the consistent trend was observed when comparing original plasma and one passaged virus stocks (Table 4). Consistent with our general impression is the decrease of sequence diversity when virus was inoculated into the mosquitoes. The hamming distance of nucleotide dropped from 2.46 to 1.7 and the number of identical clones was also increased from 15% to 31%. As shown in Fig. 5, there was a decrease in sequence diversity of original plasma compared with the C6/36 one passage cultured virus, and also increase the percentage of identical clones of original plasma compared with also the cultured virus.

| ID | No of clones | nucleotide | Amino acid |
|---|---|---|---|
| P368* | 45 | 1.405556 | 1.061111 |
| 368** | 13 | 2.461538 | 1.692308 |
| 368i^ | 28 | 1.70154 | 1.47077 |
| P388* | 20 | 2.7898 | 2.33 |
| 388** | 25 | 3.50370 | 2.87 |
| P407* | 48 | 1.039894 | 0.583333 |
| 407** | 23 | 1.905138 | 1.296443 |

*indicate sequence diversity from original plasma
**indicate sequence diversity from one passage in c6/36 cell lines of original plasma
^indicate sequence diversity from mosquito injection after one passage in c6/36 passage

Table 4. Sequence Diversity (Mean Hamming Distance) of Envelope Gene Among Different Passage Histories of Dengue Viruses.

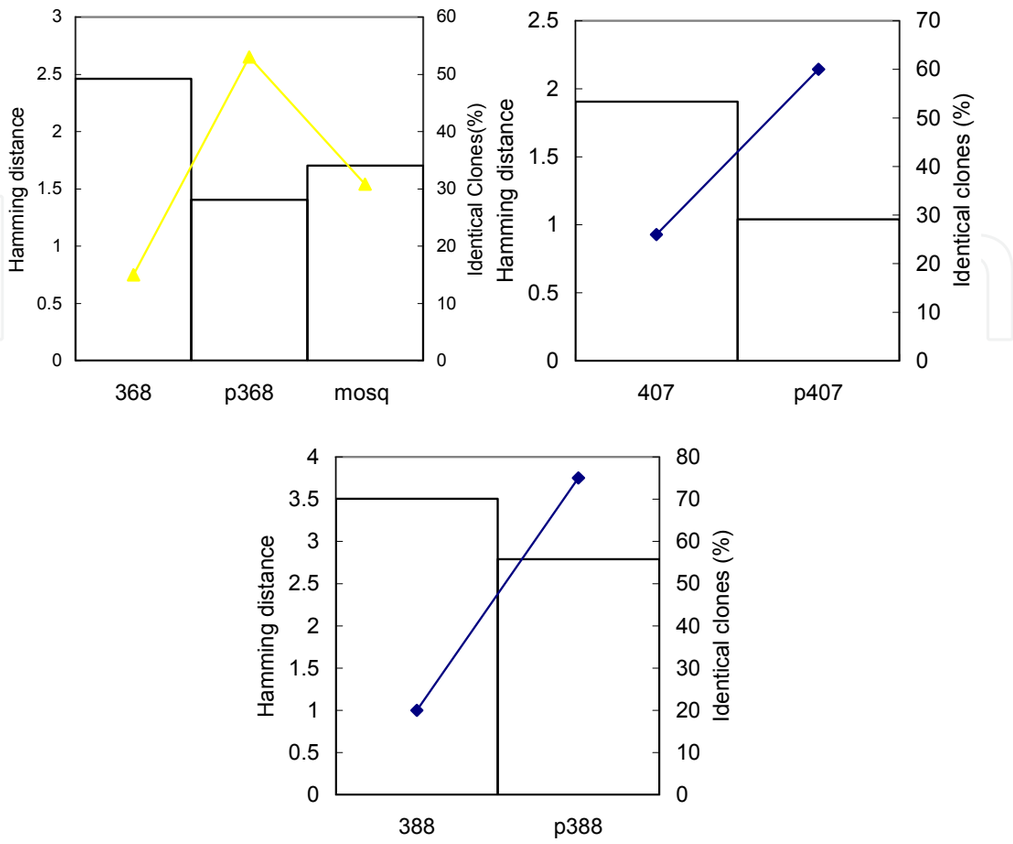


Fig. 5. Comparison of the Sequence Diversity (Hamming distance) of Nucleotide Among Original Plasma, Cultured Viruses and Mosquito Inoculation. Y-axis on the left indicated the value of hamming distance and on the right indicated the percentage of identical clones measured by the number of identical clones among total clones sequences from the isolates ID indicated at the bottom of x-axis. The value of hamming distance for individual isolates are represented as box and the value of percentage of identical clones are shown as dot and connected between isolates by solid line. (a) – (c) indicated the hamming distance and percentage of identical clones from original plasma of DHF patient, which designated by p368, p388, p407, and one passaged in C6/36 virus isolates of patients' plasma, which designated by 368, 388, 407. (a) shows the hamming distance of virus isolates from salivary gland of mosquito-*Ae. Aegypti* after intrathoracically inoculation of C6/36 passaged isolates ID368, which designated by mosq.

## 4. Discussion

Although several reports have described the uniqueness of 1998 DENV-3 epidemic where different serotypes of dengue viruses co-circulated(de Melecio, R. Barea et al. 1998; Harris, Videa et al. 2000; Rahman, Rahman et al. 2002), the epidemiology of the 1998 epidemic in non-endemic countries, including Republic of China in Taiwan has not been well-described. Without the interference of other serotypes of dengue viruses, this study allowed detailed examination of population genetics in human and mosquito hosts during intensive

transmission, which led to a better understanding of the dynamic transmission of viruses during the course of epidemic.

Most molecular epidemiological studies conducted so far have relied on consensus sequences of a virus population or an isolate. When more and more studies on the great potential for variation and phenotypic diversity of some important RNA virus pathogen, such as HIV, HCV and poliovirus have been done, it is necessary to give an appropriate set of micro-environment conditions to observe how virus quasispeices change through transmission during epidemics. Before we started to research on quasispecies of dengue viruses, it would be important to ask which region of full-genome is the most representative one for studying heterogeneity. Many viruses proposed their hyper-variable region for studying quasispecies including structure protein, like HIV V3 region of envelope protein(Delwart, Magierowska et al. 2002), HCV E2 gene (Farci, Shimoda et al. 2000; Curran, Jameson et al. 2002) and non-structure protein (NS5A) in HCV(Blight, Kolykhalov et al. 2000). What about dengue viruses? After picking up five most probable heterogeneous regions for cloning and sequencing, it was found the structural proteins especially envelope protein are more seuquence-diversed than other non-structural proteins, especially NS5 region. The structural proteins we studies here including the capsid and envelope protein. The C protein present in virions as a structure component is a small and highly positively charged protein, including N-terminal hydrophilic region, central hydrophobic region and C-terminal hydrophobic domain. The envelope protein is also the structural protein of virions plays a role in a number of biological activities including virion assembly, receptor binding and membrane fusion and is the major target for neutralization antibodies. On the other hand, the NS3 protein is the second largest viral protein, which encodes protease and helicase bi-functional protein. The C-terminal helicase protein is the region sequenced in this study. The NS5 protein undoubtly is the largest protein which role acts as the viral RNA polymerase and is the most conserved of the flavivirus protein(Chambers, Hahn et al. 1990; LinDENVVbach and Rice 2001). The larger of quasispecies among structural proteins than non-structural proteins probably implies the more selection immune pressure on the structural proteins instead of merely randomly changing during replication. This can be further validated by the data in E gene where the Domain I, the antigenic sites had larger sequences diversity than other domains. An increasing question raised upon this observation has focused on the concept that whether quasispecies distribution of dengue viruses as a reservoir of virus variants plays an essential role in diversification and selection and contribute to the dengue virus evolution.

Studies of sequence heterogeneity like our studies need to take precautions to ensure that artifacts are not introduced during the amplification of virus genomes. In this study, we used C6/36 one-passaged cultured viruses for all viruses isolates among clusters and the approach of thermostable RT-PCR kit and molecular cloning, which has been shown to be a simple and valuable method for characterization of mutant spectra of virus quasispecies. Even though this does not absolutely devoid of RT-PCR error, the relative comparison among different genomes is less biased and the linearity between E gene and other proteins should be able to trust. Furthermore, according to the study by Arias et al, the biological and molecular clones provided statistically indistinguishable definitions of the mutant spectrum with regard to the types and distributions of mutations, mutational hot-spots and mutation frequencies(Arias, Lazaro et al. 2001). Therefore, the molecular cloning procedure employed in this study provides a simple and easy protocol for the characterization of mutant spectra of viruses.

Whether dengue viruses, like other RNA viruses, exist as a quasispecies was first proved experimentally by Wang et al with nucleotide sequence diversity of the envelope gene ranging from 0.1% to 0.84% and p-distance ranging within 0.21-1.67%(Wang, Lin et al. 2002). Instead of using mean diversity represented by using the number of substitutions divided by the total number of nucleotides sequenced, our data on envelope gene used mean pairwise hamming distance ranging from 2.3 to 4.8 which is similar as being used in HCV's study(Farci, Shimoda et al. 2000). The E gene sequenced in this study contained total 1239 nucleotides and 394 amino acids, which was longer in length than previous study but similar diversity with narrower range (p-distance ranging within 0.22-0.38%). From our highly characterized family clusters, it presented consistent increase in sequence diversity from 2.3, 2.5, 3.0 in the first case (DF) to 4.8, 4.0, 3.3 in the second case (DHF) of each family cluster. Consistent with sequence diversity, the percentage of identical clones decreased from 23%, 38%, 30% in the first case to 0%, 13%, 17% in the second case in each family cluster. Further exclusion of those clones with only one single nucleotide mutation and inclusion of clones with more than two mutations, the result was the same. The percentage of identical clones decreased from 11%, 7%, 10% in the first case to 10%, 0%, 4% in the second case in each family cluster.

We hypothesize that intense transmission of dengue virus within closed environment may drive emergence of DENV-3 strain with higher propensity of causing severe diseases. It is plausible there exists at least three virus variants at any stage in mosquito or human during virus replications(Fig 6): variant M: replicate efficiently in mosquito, variant H: replicate efficiently in human, and variant N: replicate equal well in human and mosquito. We will use abbreviations at the following description. Variant N makes up the majority virus population in the quasispecies spectrum either in human, mosquito or tissue culture. Relative percentage of variant M increases when virus replicated in the mosquito; however, relative percentage of variant H increases when virus transmitted to human by mosquitoes. If virus can maintain an efficient Transovary-transmission in mosquito indefinitely, variant M will increase graduately, which might occur in the sylvatic cycle of dengue viruses and during inter-epidemic period. Variant H will increase and progeny virus or newly derived H-variant may have a higher replication capacity, thus higher virus load and higher DHF potential if human virus is transmitted mechanically from human to human through mosquito probing. Variant N possess quasispecies memory both originated from variant M and variant H. The evolution of dengue viruses comes from the random mutation accumulated in the variant N, which forms the bottleneck transmission of dengue viruses during transmission from low viremic human to mosquitoes. The possible explanation for our results is that a minor virus subpopulation with increased virulence gains rapid advantage in a direct transmission condition (ie, within family) from a certain mammalian host with peak viremia.

Several researches have been done to relate quasispecies with adaptability and host range. The alternating host cycle in arboviruses may constrain the evolution and sequence diversity among viral population. Single-host-cell adaptation by serial passage of alphavirus in mammalian cell line (BHK cell) resulted in more mutations than alternating in mammalian and mosquito cell passages(Weaver, Brault et al. 1999). So is the genetic diversity in RNA virus quasispecies, which is controlled by host-virus interaction(Schneider and Roossinck 2001). Our data also showed similar result when comparing genetic diversity among mosquito inoculates, original plasma and single passage viruses. Thus, human-mosquito-human transmission acts as a bottleneck transmission with profound fitness stability. There is also evidence showing that virus transfer might take place with high frequency between human where the donor is at

the peak of viremia(Clarke, Duarte et al. 1993; Dockter, Evans et al. 1996). Thus, the chance of a minor, more virulent virus subpopulation being transferred could have increased. The most explosive outbreak of Ross River virus-induced epidemic polyarthritis in Polynesia in the 1970s was most likely caused by the arrival of a single viraemia traveler combining with a larger susceptible population and no appropriate intermediate hosts(Bielefeldr-Ohmann and Barclay 1998). In contrast, in endemic areas pr during periods of low mosquito activity or low viraemia titers, the virus is propagated in a bottleneck transmission mode, which repeatedly selects against the variants most virulent for human.

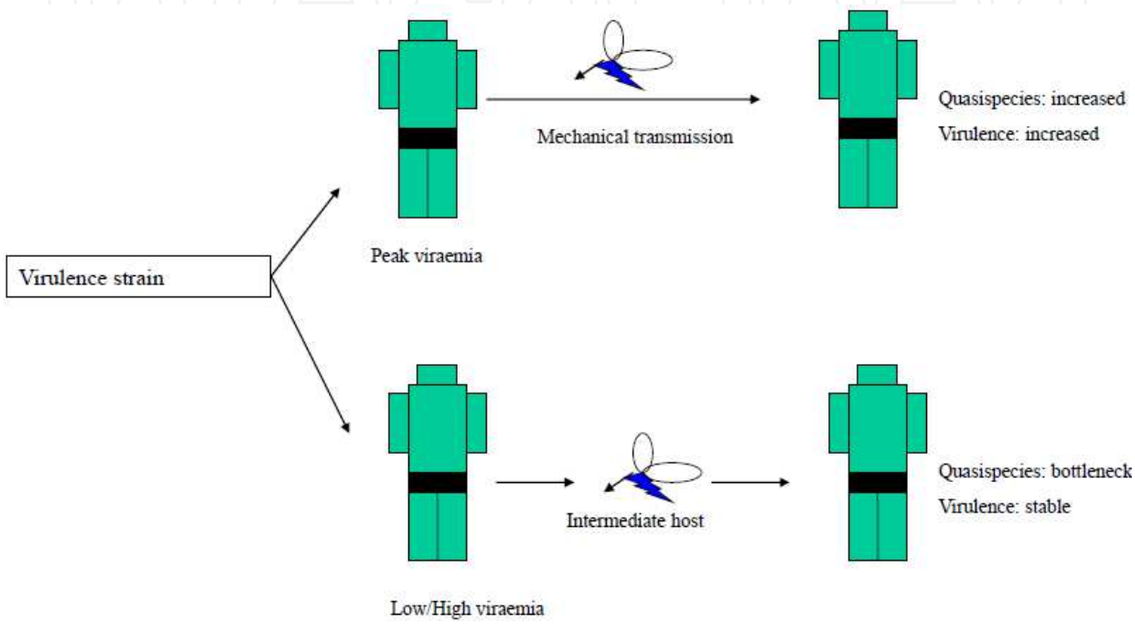


Fig. 6. Proposed model of transmission-replication mode of dengue virus and likely consequences for the quasispecies. A minor virus subpopulation with better replication capability resulting high viremia might rapidly gain selection advantage in a condition of possible direct mechanical transmission condition (ie, within a family) with peak viremia and thus provides chances to increase the viral virulence. the alternating transmission cycles of arboviruses in vertebrates and invertebrates may constrain the evolution and sequence diversity among viral populations.

Our quasispecies-based scenarios reserves a lot questions remained to be answered. First, what is the "virulence-factors" of the minor population might be? As we stated before, there is no such genetic marker in consensus sequenced or clonal sequences found in our study or in other studies. However, there exists several genetic markers repeated appearing in different isolates. Because the frequency is not high enough to say "hot-spot", those mutants reflected genomes that were dominant in the prior evolutionary history of the virus (previous passage), defining as quasispecies memory and keep to be transmitted during the epidemic(Domingo, Ruiz-Jarabo et al. 2002). Changes in virus genome may affect virus binding or replication by increasing the number of host cell receptor-specificities or binding affinities or enhance virus protein synthesis in target tissue and thus viral cytopathogenicity. Or those mutants inside quasispecies hit some viral epitopes rendering them highly inflammagenic in some genetically predisposed individuals, without following discernible

evolutionary genetic changes. With the lack of evidences of drawing conclusions based on inadequacy of animal models, the biological mechanism for changes in virulence by a replication-advantage of the mutant could be multi-factorial, which ultimately cause an imbalance in the production of components for virus entry, accumulation or assembly(Yao, Strauss et al. 1996). Also, what is the role of defective interfering (DI) particles in the pathogenesis of dengue virus infection? The involvement of DI particles has been well-studied in many RNA viruses, such as LCMV and alphavirus, which leads to virus persistence and increased cytopathology and inflammation. Our study, consistent with previous studies(Wang, Lin et al. 2002; Wang, Sung et al. 2002), also suggested the DI particles exist in human plasma with dengue viral infection. Combinations of DI particles with standard virus in the viral quasispecies leading to curing of the infection or increasing virus replication remain to be studied. Furthermore, what is the relationship of quasispecies in different compartments of the host, such as the liver, the plasma and the different immune organ play a role in dengue viral pathogenesis? In HIV, it has been suggested that different mutants in different compartments of HIV patients were associated with different tissue pathology(Marras, Bruggeman et al. 2002), as as to dengue virus which has been suggested to be able to replicate in many kinds of cells and body comparments, including liver cell, endothelial cell, dendritic cell, monocyte/ macrophage and CNS.

Finally, our study provided the most important association between genotype and phenotype made so far by observing the consistent increase in sequence diversity from the first case (DF) to the second case (DHF), which was consistent with the higher viral load in the sera of the second DHF cases of each family cluster. Thus, by preventing high viremia titer in human infection and prevent high density transmission of dengue virus, which thus decrease the viral qusispecies size and prevent severe disease manifestation will be important in public health prevention. Better understanding the evolution and quasispecies of dengue viruses with biological determinant(s) and the role of pre-existing sub-neutralizing antibody, will be crucial for future dengue vaccine.

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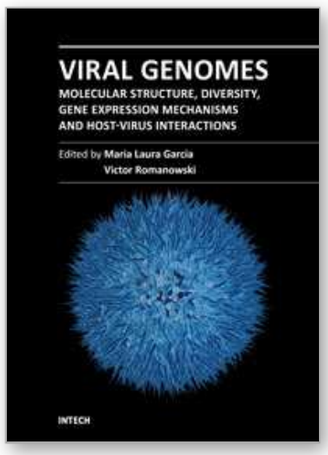


**Viral Genomes - Molecular Structure, Diversity, Gene Expression Mechanisms and Host-Virus Interactions**

Edited by Prof. Maria Garcia

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Viruses are small infectious agents that can replicate only inside the living cells of susceptible organisms. The understanding of the molecular events underlying the infectious process has been of central interest to improve strategies aimed at combating viral diseases of medical, veterinary and agricultural importance. Some of the viruses cause dreadful diseases, while others are also of interest as tools for gene transduction and expression and in non-poluting insect pest management strategies. The contributions in this book provide the reader with a perspective on the wide spectrum of virus-host systems. They are organized in sections based on the major topics covered: viral genomes organization, regulation of replication and gene expression, genome diversity and evolution, virus-host interactions, including clinically relevant features. The chapters also cover a wide range of technical approaches, including high throughput methods to assess genome variation or stability. This book should appeal to all those interested in fundamental and applied aspects of virology.

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