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DISSERTATION

MOLECULAR GENETIC INVESTIGATIONS OF DENGUE EPIDEMIC POTENTIAL AND VIRULENCE

Submitted by
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In partial fulfillment of the requirements
For the degree of Doctor of Philosophy
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Summer 2001

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JOSE ARTURO FARFAN ALE ENTITLED "MOLECULAR GENETIC INVESTIGATIONS OF DENGUE EPIDEMIC POTENTIAL AND VIRULENCE" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR IN PHILOSOPHY.

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ABSTRACT OF DISSERTATION

MOLECULAR GENETICS INVESTIGATIONS OF DENGUE EPIDEMIC POTENTIAL AND VIRULENCE

Epidemic dengue and dengue hemorrhagic fever shock syndrom (DHF-SS) have emerged as major public health problems throughout the tropical and sub-tropical world. One of the underlying reasons for this emergence is the rapid trafficking and hyperendemic circulation of dengue viruses. Introduction of virulent dengue viruses into new geographic regions has been associated with dramatic increases in DHF-SS.

Development of new molecular tools to rapidly characterize genetically dengue viruses and to detect virulent genotypes which could condition severe dengue disease was the central theme of this research.

A RT-PCR SSCP (reverse transcription-polymerase chain reaction, single strand conformation polymorphism) technique was developed to assay rapidly for genetic variability and virulence biomarkers in dengue virus genomes. Distinct regions of the genome were analyzed as genetic markers, and a sequence of the prM protein gene was the more informative region to reveal genetic diversity in dengue viruses in SSCP analyses.

RT-PCR SSCP was used to characterize dengue viruses from the Yucatan Peninsula in Mexico. Multiple haplotypes were documented in each one of the serotypes that circulated in the Yucatan Peninsula. Interestingly, dengue-3 virus exhibited the lowest genetic diversity, which is probably due to its recent introduction into the Yucatan.

RT PCR and sequence analyses were then used to investigate the effect of microenvironment on dengue virus haplotypes. Sequences of dengue virus amplified directly from a patient serum and from a virus isolate in mosquito C6/36 cells were compared. Nucleotide changes detected in virus cDNAs obtained by direct amplification from serum were predominantly synonymous substitutions. In contrast, passage in C6/36 cells yielded nucleotida changes that were non-synonymous, perhaps reflecting the selective pressures of passage in a new microenvironment.

RT-PCR was also used to investigate the possibility that virus variants that were not detectable by conventional virus bioassays could condition DHF-SS. The composition of virus populations from patients with DF and DHF were analyzed by RT-PCR SSCP. Genetic diversity was detected in each patient specimens, but there did not seem to be associations between any specific haplotype and severe dengue disease.

RT-PCR SSCP analysis is a relatively inexpensive and rapid technique for detecting genetic variability in dengue viruses. It provides a new surveillance tool to rapidly detect introduction of new and potentially virulent dengue viruses into an area. Such information could be used by resource limited public health agencies to focus control efforts in on areas posing greatest risk for severe dengue disease.

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ACKOWLEDGEMENTS

Foremost, I wish to thanks my major professor Dr. Barry J. Beaty for providing me the guidance, advice, resources and his time, necessary to complete the research work described herein. Also I wish to thanks Dr. Kenneth Olson for his continuous help and support during my time at the Arthropod-Borne and Infectious disease Laboratory (AIDL). Thanks to my remaining committee members, Drs. Carol D. Blair, William C. Black IV, Duane J. Gubler and James C. DeMartini for their helpful comments and corrections. To my wife María Alba and daughter Claudia Verónica, thanks for their support and enthusiasm. I acknowledge the financial support of the Consejo Nacional de Ciencia y Tecnología de México.

DEDICATION

To my wife María Alba and my daughter Claudia Verónica for their support and love.....

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CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Dengue fever (DF) and dengue hemorrhagic fever (DHF) cases have been reported in the tropical areas in the Americas, Asia, Australia, and Africa. Approximately 100 million cases of DF and 250,000 cases of DHF occur annually (Monath, 1995; Gubler, 1998). In the Western Hemisphere, the transmission of dengue viruses has increased dramatically in the past two decades. With the isolation of dengue-3 virus in Nicaragua in 1994, all four dengue serotypes now circulate in the Americas, and DHF has emerged as a major public health problem (Anonymous, 1995). The dramatic changes in dengue epidemiology in the Americas (Gubler and Clark, 1995) have been attributed to many factors including: the introduction of new serotypes of dengue in specific geographic areas, the decline in public health and mosquito control infrastructures, increased air travel, and urbanization (Gratz, 1999).

Historical introduction

Yellow fever was the first human disease attributed to a filterable virus, and dengue was the second. Dengue virus transmission by mosquitoes was demonstrated in 1903 in Lebanon. A few years later, *Aedes aegypti* was demonstrated to transmit the virus from one person to another after an extrinsic incubation period (Innis, 1995). The virus was first isolated in 1943 from serum of an acutely ill patient in Japan. This was the strain Mochizuki, which later was clasified as dengue 1. (Gubler, 1997). The prototype of the dengue-1 serotype (Hawaii) was isolated in 1944. The prototype of dengue-2 (New Guinea-C) was isolated in the same year (Sabin, 1952). In 1956 the other two serotypes of dengue viruses, dengue-3 and dengue-4 were isolated (Hammon *et al.*, 1960).

The first described epidemics that could have been caused by dengue viruses occurred in Cairo, Egypt and Batavia (Jakarta), Indonesia, during 1779. The following year an epidemic occurred in Philadelphia in North America. Several other outbreaks, with clinical descriptions of cases similar to dengue, occurred during the 19th and 20th centuries: in southern United States in 1922, in Greece in 1927-1928, western Australia in 1925-1926 and 1942, and in Japan 1942-1945 (Innis, 1995). New information based upon clinical descriptions suggests that dengue occurred in China before the year 1000, in French West Indies in 1635, and in Panama in 1699 (Gubler, 1997).

Clinical cases compatible with DHF were described during a major epidemic in Athens, Greece in 1928. In that epidemic 650,000 persons were ill and more than 1,000 died (Halstead and Papaevangelou, 1980; Rosen, 1986). It has been also stated that the first epidemic of DHF occurred in Charters Towers, Australia during 1897 (Mackenzie *et al.*, 1996). The first epidemics of dengue with clinically diagnosed cases of hemorrhagic fever occurred in 1954 in Manila, Philippines and Bangkok, Thailand (Hammon *et al.*, 1960). Other outbreaks occurred in the same area in the following years. Beginning in the late 1950's, the disease expanded to many other Asian and Pacific Ocean countries, and subsequently the Caribbean and the Americas.

Dengue in the Americas

The initial description of a dengue like disease in the Americas was published in 1789; an epidemic of a disease that clinically resembled dengue occurred during 1780 in Philadelphia (Halstead, 1992). During the following years, 4 major epidemics of dengue occurred (1827-1828, 1850-1851, 1879-1880 and 1897-1899), affecting several Caribbean

countries and the southern region of the United States (Ehrenkranz *et al.*, 1971). In the following years epidemics were reported in the Caribbean region in 1905-1907, 1922, 1934-1938, 1941-1946, 1963-1964 and 1968-1969 (Ehrenkranz *et al.*, 1971).

The first dengue virus isolated in the Americas was dengue-2; this virus was isolated from a patient who had DF during an epidemic in Trinidad in 1953 (Anderson *et al.*, 1956). In the 1963-1964 epidemic, which affected several countries in the Caribbean region, dengue-3 virus was isolated from a few patients in Puerto Rico (Gratz and Knudsen, 1995a). During the 1968-1969 epidemic in Jamaica, Dominican Republic, Haiti, several islands of the Lesser Antilles, and Venezuela, the co-circulation of different dengue serotypes was reported for the first time. Dengue-2 and dengue-3 viruses were isolated from patients from Jamaica (Ehrenkranz *et al.*, 1971). In Colombia, extensive epidemics were caused by dengue-2 and dengue-3 during 1971-1972 and 1975-1977, respectively (Pinheiro and Corber, 1997) . Dengue-1 was isolated for the first time in the Americas in Kingston, Jamaica during 1977 (Gratz and Knudsen, 1995b).

During 1981, dengue-4 virus caused epidemics for the first time in the Caribbean, the northern part of South America, Central America and Mexico (Pinheiro, 1989). Even though dengue-4 virus usually had been associated with a mild form of DF, it caused severe hemorrhagic cases in the Yucatan a few years later (Loroño-Pino *et al.*, 1993).

In the early 1990s, two remarkable events concerning the epidemiology of dengue in the Americas occurred. During 1993, Panama and Costa Rica, which had not reported dengue circulation during previous years, reported clinical cases of the disease. Dengue-1 and dengue-2 viruses were associated with the epidemics in those countries (Anonymous, 1994). In 1994, dengue-3 was reintroduced into the Americas; the last time this virus had

been reported was 1978. By the end of 1994, cases caused by dengue-3 virus infections were reported in Managua, Nicaragua, and subsequently dengue-3 virus isolations were reported in Panama (Anonymous, 1995). Dengue-3 virus was then detected in Mexico, (Briseño-García *et al.*, 1996). A dengue-3 virus (id. BC/96) was isolated from a patient in Mérida, Yucatán in 1995. Since 1994, the four dengue serotypes have been circulating in the Americas.

In the Americas, epidemics of DHF started in 1981 and since then, there has been an increasing trend in the number of cases and countries affected. A critical point in the development of DF/DHF in the Americas was the introduction of dengue-1 virus during 1977. Initially, cases associated with this serotype were detected in Jamaica, and then the disease expanded to many Caribbean islands. South America was also affected in the following year, and many countries reported the disease including Venezuela, Colombia, Guyana, Surinam and French Guiana. The epidemic extended to Central America during the same year, reaching Honduras, El Salvador, Guatemala and Belize. During the last months of 1978, dengue cases were detected in Chiapas in the South of Mexico. The disease disseminated to most of the states of Mexico during 1979-1980, and it reached the southern United States of America by the second half of 1980 (Pinheiro, 1989). During 1981 the first major epidemic of DHF occurred in the Americas. Prior to this epidemic, unconfirmed DHF had been suspected and reported sporadically in Venezuela, Jamaica, Honduras, Curacao and Puerto Rico (Pinheiro, 1989). Cuba experienced a major epidemic during 1981 that lasted more than 3 months. A total of 344,203 cases of dengue were reported; 10,312 were clinically classified as DHF/DSS (dengue hemorrhagic fever/dengue shock syndrome) and 158 persons died (101 children and 57 adults) (Kouri *et al.*, 1986).

The low case fatality rate was due to excellent public health intervention and effective clinical care of patients. A total of 116,000 cases were hospitalized (Kouri *et al.*, 1986). This epidemic was associated with the introduction of dengue-2 virus into Cuba and occurred four years after an epidemic of dengue-1 virus that affected most of the population of the island.

During the 1980s dengue-1 virus caused important epidemics in an extensive area of South America. Brazil, Bolivia, Paraguay, Ecuador and Peru experienced major epidemics. Dengue-1 was the virus serotype responsible, but in Peru dengue-4 virus was also isolated. The co-circulation of two serotypes was also identified in the northern area of Brazil (Pinheiro and Corber, 1997).

Another important epidemic of DHF occurred on 1989-1990 in Venezuela; 3,108 cases were diagnosed with 73 fatalities. Dengue-2 virus was the predominant serotype isolated during the epidemic, but dengue-1 virus and dengue-4 virus were also isolated. Another outbreak of DHF occurred during 1990-1991 in Rio de Janeiro in Brazil (Anonymous, 1997). Every year, more cases of DHF were reported in more countries (Pinheiro and Corber, 1997).

The epidemics of DHF in Cuba and Brazil were associated with dengue-2 virus and were preceded by epidemics of dengue-1 virus four years earlier. In contrast, in Venezuela and French Guiana dengue was endemic for about 20 years before the first epidemics of DHF occurred during 1989-1990 and 1990-1991, respectively. In both of these epidemics, dengue-2 virus was isolated (Anonymous, 1990; Reynes *et al.*, 1994).

After the 1981 epidemic of DHF in Cuba, an intensive campaign was established to control and eradicate *Aedes aegypti* from the island. Eradication was not achieved, but

control of the vector was successfully implemented and sustained for several years. Most of the Cuban municipalities were free of the vector. However, after 15 years of being dengue free, another epidemic of dengue occurred in Cuba during 1997. A total of 2,946 cases of dengue were confirmed, including 205 cases of DHF with 12 fatalities. Dengue-2 virus was isolated from 46 cases (Kouri *et al.*, 1998).

Dengue Fever and Dengue Hemorrhagic Fever.

Two major clinical forms of dengue, dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) have been described. The clinical spectrum of DF is characterized by a multiplicity of clinical manifestations, the more common are: fever, headache, myalgia, arthralgia, retro-orbital pain, photophobia and in some cases pruritus in palms and soles. About 30% of the patients have a maculopapular rash. In some of the patients, pruritus specially on palms and soles may follow the exanthema. Adenomegalia is not common but present in some cases. The disease is self limited and the patient usually is free of symptoms in 5 days to a week (Nimmannitya, 1987). DF is often confused clinically with other viral diseases like measles, rubella, enteroviruses and influenza (Waterman and Gubler, 1989).

A more severe form of the disease is DHF. In addition to the signs and symptoms described for DF, loss of intravascular fluids occurs. The most dramatic presentation of DHF is the presence of hemorrhage. However, loss of intravascular fluid without bleeding is frequent. Plasma fluid leakage through vessel walls and accumulation in tissues or natural cavities is considered the pathognomonic for DHF (Innis, 1995; Rothman and Ennis, 1999). Loss of fluids typically starts when fever decreases (George and Lum, 1997). The World Health Organization has established four grades for the severity of DHF.

The characteristics of the four grades of DHF, as stated by WHO (World Health Organization, 1997) are:

- Grade I: with fever and non-specific constitutional symptoms. The only hemorrhagic manifestation is a positive tourniquet test and/or easy bruising.
- Grade II: spontaneous bleeding in addition to the manifestation of Grade I patients, usually in the forms of skin or other hemorrhages.
- Grade III: circulatory failure manifested by a rapid, weak pulse and norrowing of pulse pressure or hypotension, with the presence of cold, clammy skin and restlessness.
- Grade IV: profound shock with undetectable blood pressure or pulse.

Grades III and IV are considered dengue shock syndrome (DSS). Bleeding can be present in several forms, such as severe epistaxis, hematemesis, petechiae, ecchymosis, internal bleeding, gastrointestinal bleeding, menorrhagia, metrorrhagia, and menometrorrhage. Drastic accumulation of fluid in the pleural and pericardial space can also be present. In DHF grades II to IV the number of platelets is typically below 100,000 per mm^3 and the hematocrit value is typically $\geq$ 20% over the normal value. Uncommon clinical presentations include severe hepatic failure or encephalitis (Nimmannitya *et al.*, 1987; Mehendale *et al.*, 1989; Lum *et al.*, 1996; Row *et al.*, 1996; Hommel *et al.*, 1998; Couvelard *et al.*, 1999). In most of these cases, the interpretation has been that the liver or encephalitic afflictions are secondary to the hemorrhagic process. However a case of classical DF with neurological complications that started during the acute phase of the disease, (only two days after the disease onset) was recently reported (Hommel *et al.*, 1998). Dengue virus was isolated from CSF and dengue-2 virus sequences were amplified

from serum and CSF.

Two principal hypotheses have been proposed to explain the increased severity of clinical symptoms occurring in DHF: the antibody dependent enhancement (ADE) of infection theory and the more virulent virus variant theory. Both theories are supported by epidemiological data. Both host and viral factors may participate in the pathogenesis of DHF. The host immune response may facilitate viral infection of cells, which may be the basis of the antibody dependent enhancement hypothesis (ADE) (Halstead, 1988; Morens, 1994). ADE is typically associated with secondary or sequential infection with different dengue serotypes. Infection with one dengue serotype does not protect from infection with a different serotype. However, non-neutralizing antibodies to the first virus may bind to the second virus. This virus-antibody complex is then bound by the Fc part of the antibody to the Fc receptor of monocyte cells and internalized in a highly efficient process (opsonization), which can result in different tissue tropism and pathogenesis (Tio and Malasit, 1995). In addition, virus-antibody complexes may activate the complement system (Malasit, 1987), resulting in T cell based immunopathology (Kurane *et al.*, 1994).

Infected cells become targets for cell mediated cytotoxic events. The infected and effector cells induce cytokines and other soluble mediators eg. C3a, inteferon-gamma, Interleukin-1 and interleukin-2 (Kurane *et al.*, 1994; Halstead, 1997). The release of these chemical mediators increases the vascular permeability (Kurane *et al.*, 1994, 1995; Innis, 1995; Rothman and Ennis, 1999). It has been proposed a model that incorporates several of the components of the immune response in the pathogenesis of DHF. This model includes the synergistic interaction of interferon γ (IFN-γ), tumor necrosis factor α (TNFα)

and activated complement proteins on the endothelial cells, as the cause of capilary leakage in secondary infections. In the early stage of the infection, antibodies that were produced in a previous infection increase the number of infected monocytes. This results in a increased number of cells presenting dengue antigen to T lymphocytes. Activation of T lymphocytes is then increased an also the proliferation of memory T lymphocytes. T lymphocytes produce several cytokines such as IFN-γ, TNFα and TNFβ and interleukin-2. These cytokines may induce capillary leakage through multiple direct and indirect effects in the vascular endothelium (Rothman and Ennis, 1999).

In support of the first theory, most cases of DHF occur in patients having a secondary infection with a different serotype of dengue. Epidemiological observations provide support for the ADE hypothesis. For example during the epidemic in Cuba in 1981, almost all the patients who had DHF were experiencing secondary infections with dengue-2 virus. They had presumably been infected with dengue-1 virus four years previously in 1977 (Guzman *et al.*, 1984).

Evidence of ADE effect has been also supported by observations based maternal antibodies to dengue. If the mother has antibodies resulting from infection with one or more dengue viruses, these broadly neutralizing antibodies will be passively transferred and will protect the neonate against dengue infection during the initial months of life. However, natural catabolism will reduce progressively the antibodies in the newborn in the following months. Once maternal antibodies are no longer protective, the newborn will be subject to ADE effect when infected with a dengue virus and increased risk for DHF/DSS (Halstead, 1981). ADE has been produced in vitro using mouse cell lines and rabbit

antibodies (Halstead *et al.*, 1983).

ADE has also been observed *in vitro* with other viruses such as HIV-1, influenza A, BK papovavirus and feline infectious peritonitis (Burke, 1992; Mascola *et al.*, 1993; Halstead, 1994). In dengue, most secondary infections only result in DF; only a small proportion of secondary infections (0.5% to 5%) result in DHF (Sangkawibha *et al.*, 1984; Burke *et al.*, 1988).

Infection with more virulent strains of viruses may also condition DHF. Whith this hypothesis, more virulent virus genotypes occur in the population of viruses present in nature and may be the cause of DHF.

The virulent virus hypothesis for DHF is supported by epidemiological investigations of epidemics associated with only one virus serotype, and DHF has been reported in patients experiencing primary infections (Barnes and Rosen, 1974; Scott *et al.*, 1976; Kuberski *et al.*, 1977; Morens *et al.*, 1987; Gubler *et al.*, 1978). In addition, dramatic differences in the intensity of the epidemics or clinical manifestations in the populations affected have been associated with a different viruses of a given serotype (Gubler, 1988).

There is other evidence that suggests that viral genotypic features may condition virulence. In Thailand during 1980, about 20% of children who had secondary infections caused by dengue-2 virus developed DHF (Sangkawibha *et al.*, 1984). In contrast, in a study conducted in Iquitos, Peru, 0% of 49,266 school children who had dengue-2 secondary infections developed DHF (Watts *et al.*, 1999).

Clearly some strains of dengue viruses have been associated with more severe epidemics. For example the Puerto Rican strain of dengue-2 virus was present in many countries in the Americas for many years without causing severe disease. However when

a different strain of dengue-2, the Jamaica strain (an Asian genotype) was introduced into the Americas, major epidemics of DHF occurred in Cuba, Venezuela and Brazil (Rico-Hesse, 1990; Gubler and Clark, 1995).

Molecular epidemiology studies have indicated that the "Asian" haplotypes of dengue viruses that are progressively being introduced into the Americas are replacing the pre-existing genotypes. The replacement of the "American" genotypes by the "Asian" genotypes has been associated with the increase in the severity of the disease in several countries in the Americas (Trent *et al.*, 1983; Rico-Hesse, 1990).

Nucleotide sequence data analysis also suggest that some dengue viruses have a greater virulence and that this is associated with specific nucleotide sequences in their genome. This information is presented in more detail in the section entitled "Dengue virus genetic determinants of virulence" (page 22).

It is possible that competition between related dengue viruses selects for genotypes that have higher replication rates, higher fitness or higher virulence in vectors or vertebrate hosts (Frank, 1992; Nowak and May, 1994). Dengue epidemics in the Americas currently seem to be more severe than those that occurred in previous years. Dengue has become hyperendemic in many parts of the world. This could lead to introduction or generation of new virulent strains, increased likelihood of secondary infections, and much more DHF.

Humans are now in more frequent contact with dengue viruses of different serotypes and genotypes than in the past (Zanotto *et al.*, 1996). These interactions could increase intramolecular evolutionary events in the viruses (eg. point mutations, translocations and potentially recombination events, etc). Viral recombination represents

a different mechanism that could increase the genomic diversity in dengue viruses. Dengue virus can apparently recombine in nature (Holmes *et al.*, 1999; Worobey *et al.*, 1999). A group of 71 dengue virus isolates were analyzed and 7 recombinants were identified (Worobey *et al.*, 1999). Epidemics in which different dengue virus serotypes co-circulate has increased in recent years, and patients with concurrent infections with more that one serotype have been reported (Gubler *et al.*, 1985; Laille *et al.* 1991; Lorono-Pino *et al.*, 1999). Recombination could contribute greatly to the evolutionary potential of dengue viruses. Host genetic factors could also condition a more severe dengue disease in some populations. A few investigations have suggested the possibility of association of specific HLA alleles and increased susceptibility for DHF. However those studies are limited and the results do not agree, possibly because they were conducted with populations with different genetic background (Chiewsilp *et al.*, 1981; Bravo-Gonzalez *et al.*, 1985; Paradoa Perez *et al.*, 1987; Rothman, 1997).

The transmission cycle

Dengue viruses are maintained and amplified in nature in mosquito-primate cycles. Transmission of dengue viruses occurs when infected female *Aedes* mosquitoes bite susceptible primate hosts (Gubler, 1988). In tropical Asia and in West Africa, a forest cycle of dengue virus transmission has been demonstrated involving non human primates and forest mosquitoes. It is not clear what relationship exists between this forest cycle and the transmission of the viruses to humans (Monath, 1994a). Recently, serologic evidence indicating active virus transmission was demonstrated among macaques in Sri Lanka (de Silva *et al.*, 1999).

Aedes aegypti is the principal urban vector of the dengue viruses and is the vector most frequently associated with epidemic transmission worldwide. *Ae. albopictus* is also a vector of dengue viruses in Asia and has been considered primarily as a rural or sylvatic vector (Gubler, 1988). *Ae. aegypti* is very abundant in most tropical regions of the world. It is highly domesticated and anthropophilic. It breeds in containers where water is stored for drinking or washing and in almost any container that will retain water, eg. flower vessels, tin cans, disposed plastic and glass containers, old tires, animal water dishes, buckets, etc. Such containers are frequently found in around homes, thus bringing vectors and vertebrate hosts together (Winch *et al.*, 1992).

After the infection of a susceptible person, there is an intrinsic incubation period, which ranges from 4.5 to 7 days, before the onset of symptoms (Kuno, 1995). The viremic phase usually lasts for about five days after disease onset (Hammon, 1973), during which feeding mosquitoes can be infected. There is then an extrinsic incubation period in the vector of 11 to 14 days, after which virus can be transmitted via saliva to a new host (Kuno, 1995). Once infected the mosquito is infected for the rest of its life. The longevity of the mosquitoes in nature ranges between 8 to 42 days (Trpis and Hausermann, 1986).

Virus-vector interactions

After a mosquito takes an infectious blood meal, the virus has to overcome the midgut infection barrier (MIB) to establish an infection in the midgut. Once in the midgut epithelium, the virus replicates and needs to pass a second barrier, the midgut escape barrier (MEB) to disseminate the infection. Once into the hemocel of the mosquito the virus replicates in other tissues before it infects the salivary glands, when it can be transmitted via the saliva to the next vertebrate host.

There is considerable variability in the vector competence of *Aedes aegypti* for flaviviruses (Gubler *et al.*, 1979a). Genetic groupings of *Aedes aegypti* based on allozyme analysis correlated with susceptibility for yellow fever virus (Tabachnick *et al.*, 1985). *Ae. aegypti* obtained from different regions in the tropical and subtropical regions exhibit diverse levels of vector competence (Gubler *et al.*, 1979a; Tabachnick *et al.*, 1985). These observations suggested a genetic basis for this variation in susceptibility. At least two sets of genes, whose expression where modulated by environmental factors, were demonstrated by quantitative genetic analysis of vector competence in *Ae. aegypti* for dengue-2 virus (Bosio *et al.*, 1998). Susceptibility of vectors to the infection with viruses is the result of genetic factors under the control of several loci. Vector competence is also influenced by environmental factors. Quantitative trait loci (QTL) that affect the ability of *Ae. aegypti* to become infected by dengue-2 virus via a midgut infection barrier have been mapped on chromosomes II and III (Bosio *et al.*, 2000). Different subspecies of *Ae. aegypti* differ markedly in susceptibility to viruses. Susceptibility to infection by dengue-2 virus differs between *Ae. aegypti* and *Ae. aegypti formosus*. Major determinants of vector competence for dengue viruses in *Ae. aegypti* are the barriers to midgut infection and dissemination. Those barriers are independent of virus titer in the infectious meal (Bosio *et al.*, 1998). Geographic variability in vector competence has been also reported in *Ae. albopictus*, the secondary vector of dengue (Gubler and Rosen, 1976; Tesh *et al.*, 1976). Under laboratory conditions, *Ae. albopictus* is a more efficient vector for dengue virus transmission than *Ae. aegypti*. Interestingly, however *Ae. albopictus* has been associated less frequently with major epidemics and DHF cases than *Ae. aegypti*. These observations have been the basis for the hypothesis that *Ae. aegypti* is the principal vector associated

with DHF. Because it is a less competent vector than *Ae. albopictus*, virus passage in *Ae. aegypti* would select for viral strains that produce high titered viremias. Persons with low titered viremia would not be infectious to the vector. According to this hypothesis, viral strains that produce high viremias are more virulent and more frequently associated with clinical cases of DHF and evidence supporting this hypothesis was recently published (Vaughn *et al.*, 2000).

Vector and virus virulence

Passage in insect vectors could modify the virulence of arboviruses. Epidemiologic observations of dengue epidemics that took place in several islands in the Pacific Ocean during 1971 and 1972 provided evidence to support this possibility. During those years, dengue-2 virus epidemics occurred in Tahiti, New Caledonia, American Samoa and Niue Island. In Tahiti and New Caledonia a few severe and fatal cases occurred and the incidence of the disease was low. Also in 1972, an epidemic of dengue caused by dengue-2 virus occurred in American Samoa; no serious cases of hemorrhagic diseases or deaths were reported. The same year the dengue-2 virus epidemic in Niue was severe with many cases with hemorrhagic disease and fatalities(Barnes and Rosen, 1974; Gubler 1987). During the time of the epidemics, the more abundant vector in Tahiti, New Caledonia and American Samoa was *Ae. aegypti.* In Niue the more abundant vector was *Ae. cooki* (Barnes and Rosen, 1974). The investigators speculated that this mosquito in some way could have influenced the virulence of the viruses (Barnes and Rosen, 1974).

Dengue viruses

Dengue viruses are members of the genus *Flavivirus*, family Flaviviridae. Their genome is composed of a positive-sense, single-stranded RNA approximately 11,000

nucleotides (nt) in length. The genome length varies between the different serotypes of the viruses; dengue-1, 2, 3 and 4 are 10,718, 10,723, 10,696 and 10644 nt, respectively (Zhao *et al.*, 1986; Irie *et al.*, 1989; Osatomi and Sumiyoshi, 1990; Fu *et al.*, 1992). The 5' end of the genome is capped, and there is no 3'-poly(A) tract. The 5' and 3' ends of the genome have noncoding regions of about 100 and 500 nt, respectively. The genome contains a single open reading frame and encodes a single polyprotein that is cleaved co- and post-translationally to produce 10 viral proteins in this order : 5' -capsid (C), premembrane (prM), envelope (E), nonstructural protein 1 (NS1), NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Chambers *et al.*, 1990; Henchal and Putnak, 1990). The 5' and 3' terminal non-coding regions contain one and two conserved sequences, respectively (5' CS and 3' CS2, CS1). CS2 is the most highly conserved sequence among flaviviruses and is duplicated in members of the JE and dengue subgroups (Chambers *et al.*, 1990). The 3' end forms a stable hairpin loop structure.

The capsid protein (C) is the least well conserved of all the virus structural proteins; it is rich in proline and has no cysteine residues, which permits a high degree of conformational freedom. The mature form of the virion, both intra and extracellular, lacks about 14 amino acids of the carboxyl end of the protein. The C protein has been detected in the cytoplasm and also in the nucleus of infected cells (Tadano *et al.*, 1989).

The nucleocapsid is surrounded by a lipid bilayer in which the membrane (M) and envelope (E) proteins are immersed. A glycosylated precursor of the M protein (prM) is cleaved during the virus maturation, resulting in the production of the M protein, which is present in the mature form of the virion. The cleavage occurs at a specific site, which is

conserved among flaviviruses, before the virus is released to the extracellular space. This cleavage of prM is important also for the infectivity of the viral particle. Viruses in which prM cleavage does not occur are less infectious than those in which the cleavage takes place (Henchal and Putnak, 1990). Based on studies on tick-borne encephalitis, the prM protein probably functions to protect the E protein from irreversible acidic pH-induced conformational changes of that protein during exocytosis (Heinz *et al.*, 1994). A hydrophobic sequence of about 40 amino acids in the carboxyl end is inserted in the virus lipid bilayer envelope. An amino terminal region about 40 amino acids long is exposed on the external surface of the virion (Markoff and Falgout, 1995).

The E protein is also inserted in the lipid envelope, is about 500 amino acids, and is glycosylated. It has a number of critical viral functions have been associated with it. E possesses the major antigenic sites of the virus, is the virus attachment protein, and has hemagglutination and fusion activity (Summers *et al.*, 1989; Randolph and Stollar, 1990; Markoff and Falgout, 1995). There are twelve positions with cysteine residues that are highly conserved among flaviviruses. These residues are important for the formation of disulfide bonds (Mandl *et al.*, 1989a; Rey *et al.*, 1995). The E protein is a monomer with two transmembrane segments at its carboxyl-terminal end. Rather than forming a spike-like structure, it extends parallel to the viral surface. The E protein forms dimers on the surface of mature virion at physiological pH (Rey *et al.*, 1995) .

Virus assembly takes place at the membrane of the endoplasmic reticulum (ER) and leads to the formation of immature particles containing prM/M, C and E proteins. Viral particles pass from the ER to the Golgi apparatus. PrM/M forms a complex with protein E during virus maturation (Heinz *et al.*, 1994). Cleavage of prM/M occurs during exocytosis,

probably by a furin-like protease. Immature virions do not exhibit low pH induced conformational change or fusion activity. This observation is interpreted as evidence of the function of prM in the prevention of irreversible inactivation during the transport of the virus through the acidic environment that prevails in the trans Golgi network (Chambers *et al.*, 1990; Heinz *et al.*, 1994; Rey *et al.*, 1995).

The non structural proteins are NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Chambers *et al.*, 1990; Henchal and Putnak, 1990). NS1 is a protein that has been shown to be inserted in the membrane of the infected cells. NS1 protein can be found in monomeric or dimeric forms, however the functional form of the protein is the dimer (Winkler *et al.*, 1988). Antibodies against this protein can provide protective immunity. This protection could be due to interaction of antibodies with NS1 in the cell membrane and destruction of the infected cell. NS1 protein may be important in the process of maturation of the virion (Fan and Mason, 1990). Studies with yellow fever virus suggest that NS1 protein also participates in RNA replication (Muylaert *et al.*, 1997; Lindenbach and Rice, 1999).

NS2A and NS2B code for two proteins, which are poorly conserved among Flaviviruses. NS2A is important for the cleavage of the viral polyprotein in the NS1-NS2A junction (Falgout *et al.*, 1989). NS2B complexes with NS3 protein to form a protease that cleaves basic sites on the viral polyprotein (Arias *et al.*, 1993; Chambers *et al.*, 1993).

NS3 is highly conserved among Flaviviruses and is the largest protein after NS5. This protein has several functions. When complexed with NS2B the amino terminal portion has protease activity. In the same protein, three nucleoside tri-phosphatase and helicase activities have been identified (Wengler and Wengler, 1993; Warrener and Collett, 1995;

Kuo *et al.*, 1996; Tai *et al.*, 1996).

NS4A and NS4B functions are not well known. NS5 is the largest protein coded by the flavivirus genome and is also the most conserved. It is believed to be the RNA dependent RNA polymerase (Mandl *et al.*, 1989b; Bartholomeusz and Wright, 1993). This observation is based on the existence of a highly conserved region that includes the sequence motif GDD, which is characteristic of known RNA-dependent RNA polymerases of positive-strand RNA viruses (Rice, 1996). NS5 protein may have methyltransferase activity in addition to RNA dependent RNA polymerase activity.

Virus phylogeny

Phylogenetic analysis of 123 complete envelope gene sequences suggested that Flaviruses emerged within the last 10,000 years and the dengue viruses diverged from the other flaviviruses about 2,000 years ago (Zanotto *et al.*, 1996). There are 4 serotypes of dengue viruses. It has been postulated that divergence of serotypes occurred in relatively recent time. Phylogenetic analysis reveals that among flaviviruses an initial separation occurred between tick and mosquito-borne viruses; the mosquito-borne flaviviruses then divided into several clades, the yellow fever, Japanese encephalitis (JE) and dengue viruses. In the dengue viruses, initial divergence occurred with dengue-4 virus, followed by dengue-2 virus and then dengue-1 virus and dengue-3 virus (Zanotto *et al.*, 1996). In both the dengue and JE groups, the time of intense phylogenetic divergence apparently occurred during the last 200 years (Zanotto *et al.*, 1996). Phylogenetic analysis suggests that for a long time dengue viruses had a very limited number of lineages. However, the generation of new lineages increased dramatically in the more recent years. As viral genetic diversity increases, chances increase that new phenotypic characteristics could

result, such as greater virulence or transmissibility, faster replication in mosquito vectors or humans, or even adaptation to alternate vectors (Holmes, 1998).

The nucleotide sequence of the prM and E genes of dengue viruses has been used extensively for phylogenetic analysis of virus isolates in the same serotype. Analysis of a 250 nt region at the junction of E and NS1 genes revealed the existence of 5 distinct genotypes of dengue-1 virus (Rico-Hesse, 1990). Sequence analysis of the E protein gene has revealed also 5 distinct genotypes of dengue-2 virus (Lewis *et al.*, 1993). A similar analysis of genes coding for prM, M and E proteins defined 4 genotypes in dengue-3 virus (Lanciotti *et al.*, 1994). In the case of dengue-4 virus, 2 genotypes were clearly defined by nucleotide sequence analysis of the E gene (Lanciotti *et al.*, 1997).

Tissue tropism

A number of viruses in the Flaviviridae family are neurotropic. They can cross the blood-brain barrier and infect the central nervous system (CNS). CNS tissue tropism may be a characteristic of ancestral flaviviruses (McMinn, 1997). These viruses include Saint Louis encephalitis (SLE), Japanese encephalitis (JE), tick borne encephalitis (TBE), etc. However, dengue and yellow fever viruses do not typically exhibit CNS tropism. These viruses replicate in the reticuloendothelial cells and hepatic parenchyma, respectively (Monath, 1994b; Marianneau *et al.*, 1998). Both dengue viruses and yellow fever virus can be hepatotropic, but dengue viruses induce only limited foci of necrosis in the liver instead of the more dramatic effect caused by yellow fever virus (Marianneau *et al.*, 1998). Dengue virus has been recovered from liver, spleen, lung, kidney and brain (Rosen *et al.*, 1989). Dengue antigen has been identified in foci of liver necrosis (Fresh *et al.*, 1969) and in Kuppfer cells (Hall *et al.*, 1991). Dengue antigen has been demonstrated in the cytoplasm

of phagocytic mononuclear cells from liver, spleen, lung and in hepatocytes (Miagostovich *et al.*, 1997). Although most dengue virus do not tipically affect the CNS, phagocytic cells with dengue antigen have been found in white and gray matter of the brain (Miagostovich *et al.*, 1997). Dengue virus nucleotide sequences have been amplified by RT-PCR reactions and dengue viruses have been isolated from cerebrospinal fluid (Lum *et al.*, 1996; Hommel *et al.*, 1998; Ramos *et al.*, 1998). Dengue antigen has also been observed in neurons, astrocytes, microglia and endothelial cells in the brain (Ramos *et al.*, 1998).

Dengue virus genetic determinants of virulence

A direct relationship between dengue disease severity and virus load had been hypothesized (Halstead, 1988; Gubler 1983; Gubler, 1987). The theory was that viral strains producing high viremias would be associated more frequently with clinical cases of DHF; in contrast, viral strains producing low viremia, would cause a lower proportion of clinical cases or clinical cases with milder symptoms (Gubler, 1987). Recently, this suggestion has been confirmed. Higher peak dengue virus titers were associated with more severe disease (Vaughn *et al.*, 2000).

The theory of viral strains with enhanced virulence has been supported by the observation of specific nucleotide sequences in the viral genome that have been associated with severe clinical presentations. These observations have motivated studies oriented to analyze the nucleotide sequence of dengue virus isolates obtained from DF and DHF patients. The possibility that mutations in the viral genome associated to amino acid changes that modify the natural function of viral proteins have been considered. Non synonymous nucleotide substitution is one occurring in a protein-coding region and resulting in an amino acid change. Synonymous substitution does not result in amino acid

change. Several observations have been reported with virus isolates obtained from DHF cases. Nucleotide sequence comparisons of the envelope glycoprotein gene of dengue-2 virus from three patients from Thailand with different degrees of severity of dengue (DF, DHF and DSS) were conducted (Duangchanda *et al.*, 1994). The deduced amino acid sequence from the three isolates was identical and thus no association between the E sequence and disease severity was revealed (Duangchanda *et al.*, 1994). A similar analysis was conducted with dengue-2 strains obtained from four patients from Thailand with different disease severity. In this study, all of the structural proteins: C-prM(M)-E and the non structural protein NS1 were analyzed and their nucleotide and amino acid sequences were compared. The nucleotide sequences of the C protein was highly conserved among the four isolates (1.75% to 2.63% divergency), in constrast the nucleotide sequences of the prM region showed the highest divergency (6.59% to 7.32 %). One amino acid change at position 130 in prM did result in a change from a positive charged amino acid to a non polar one, and this was associated with disease severity. The isolate from the DF case had an arginine at 130, but all isolates from DHF cases (two isolates from DHF and one from DHF/DSS patients) had isoleucine. Thus the nucleotide and amino acid changes were associated with the virulence of the isolates (Thant *et al.*, 1996).

A similar analysis of a 240 nucelotides sequence of the E/NS1 gene junction of dengue-2 isolates from patients with different clinical severities was conducted. These isolates were obtained during the 1987 epidemic in Yangon, Myanmar. Two isolates were obtained from patients with DSS and one from another patient with DHF grade I. Two and three nucleotide differences were found when comparing the DHF with the DSS isolates.

However, all the substitutions were in the third position of the codon, and thus did not result in changes in the amino acid sequences (Thant *et al.*, 1995a). The same genomic region was analyzed in dengue-2 virus isolates obtained from patients with different clinical severities during the 1993 epidemic in Nakhon Phanom in the northeast of Thailand. Again, all nucleotide substitutions found were located at the third position in the codon (Thant *et al.*, 1995b).

Subsequently, the whole genomes of dengue-2 viruses isolated from eight patients exhibiting different disease severities during an epidemic in northern Thailand during 1993 were analyzed (Mangada and Igarashi, 1998). Thirty amino acid replacements were found among the isolates. They were scattered throughout the entire genome, but most were located in the non-structural region. The only isolate obtained from a patient with DSS had the greatest nucleotide divergence (across the whole genome) of all the isolates. Interestingly seven amino acid substitutions were unique to the isolate from the DSS patient, all of which were located in the non structural region (Mangada and Igarashi, 1998).

Molecular epidemiologic studies have associated certain virus genetic types with severe dengue epidemics, which could indicate strains with increased pathogenicity (Rico-Hesse *et al.*, 1997). Nucleotide sequences (240 nt) from the E/NS1 gene region of the dengue virus genome were compared. This study revealed association between the introduction of two genotypes of dengue-2 and the increase of DHF in the Americas. Phylogenetic analysis indicated that the new genotypes originated in Southeast Asia and that they are displacing the native American genotype, which has been associated to less severe manifestations (Rico-Hesse *et al.*, 1997; Rico-Hesse *et al.*, 1999; Watts *et al.*,

1999).

Dengue-2 virus was isolated from a blood sample from a 12 year old boy who died of DHF after having a clinical presentation of dengue in which encephalitic manifestations were predominant. The case occurred during the 1991 epidemic season in Thailand. A fragment of the E gene was sequenced from the viruses isolated from the patient and from two other patients, who also had DHF during the same epidemic, but who did not present manifestations of CNS infection. The virus isolated from the patient who had encephalopathy differed from the other two isolates in a single amino acid. The substitution was in position 173 of the E protein, resulting in valine instead of alanine (Sistayanarain *et al.*, 1996). This change was in the G_0 region of the E protein (Rey *et al.*, 1995). The amino acid substitution was a conservative replacement and one that was not previously associated with changes in virulence (Sistayanarain *et al.*, 1996). Conservative mutations are those that result in a codon being replaced by another codon that specifies a different amino acid, but one that is related in chemical properties to the original coded amino acid.

In vitro and animal studies also suggest specific genetic determinants of virulence. Specific changes in the nucleotide sequence of the prM gene were associated with enhanced virulence of dengue viruses (Thant *et al.*, 1996). Large and small plaque forming variants of dengue viruses caused different levels of mortality in mice that were intracerebrally inoculated. Specific nucleotide sequences in the E coding gene were associated with the increased mortality rate in mice (Sanchez and Ruiz, 1996).

Using a lytic plaque selection system, dengue-2 virus clones producing large and small plaques were selected. Large plaque clones were highly virulent for suckling mice

when injected intracerebrally, causing ≥ 70% mortality; in contrast virus clones that produced medium size and small plaques caused 0 to 20% mortality. Sequence analysis of the envelope protein gene (E) and its corresponding amino acid sequence revealed that the large size plaque clone differed from the parent virus by 11 nucleotides, but 10 of these resulted in silent mutations. A substitution at position 1168, G→C, resulted in a non conservative amino acid change, asparagine →histidine, replacing a negatively charged amino acid with a positively charged one. In small size plaque clones, which were less virulent for suckling mice, a replacement in the same position resulted in a conservative change in the amino acid sequence: aspartic acid→ asparagine. The low virulence mutants also had less overall nucleotide replacements than the highly virulent viruses. The low virulent viruses had a conservative substitution in position 1168, and a non-conservative substitution in position 1164. This substitution resulted in a lysine→asparagine amino acid change (positively charged to negatively amino acids). The non conservative change at position 1168 was associated with enhanced virulence and large size plaque phenotype (Sanchez and Ruiz, 1996).

Sequence of the C-PrM-E-NS1 region of dengue-1 (strain NGC) and its mouse neurovirulent mutant generated by 38 serial intracerebral passages in mice were compared. This mutant virus is highly neurovirulent for mice but attenuated for humans. Ten nucleotide changes were revealed between the parent and progeny viruses. Seven of these changes were non synonymous. The sequence of gene C was highly conserved. In the other genes the changes were as follows: in prM leucine→phenylalanine in position 55, arginine→lysine in 57, in E glutamic acid→aspartic acid in 71, glutamic acid→lysine in

126, phenylalanine→isoleucine in 402 and threonine→isoleucine in 454. In NS1 arginine→glutamine in 105. Analysis of the amino acid replacements indicated that glutamic acid 71→aspartic acid and glutamic acid 126→Lysine changes, both of them in the E gene, conditioned the neurovirulence. The glutamic acid 126→lysine change, which substituted a negatively charged amino acid for a positively charged one, was especially important. The glutamic acid 71→aspartic acid change was conservative but may have had a minor effect (Bray *et al.*, 1998).

Identification of viral molecular markers of pathogenicity is important to understand how viruses affect host cells, and also for vaccine development. For dengue, several studies have been conducted, but no consistent results have been obtained. However sites located in prM and E protein coding regions have been suggested as possibly associated with virulence (Thant *et al.*, 1996; Sanchez and Ruiz, 1996; Sistayanarain *et al.*, 1996; Rico-Hesse *et al.*, 1999).

RNA virus evolution

Viruses with RNA genomes are the most ubiquitous cellular parasites, infecting almost all life forms. It has been estimated that more than 70% of viruses that infect diverse organisms have RNA genomes (Domingo and Holland, 1994). Because they are responsible for a variety of medically and economically important diseases of humans, animals and plants, they have been studied extensively.

The evolutionary success of RNA viruses is attributable to the ability of these viruses to use different replication strategies and to adapt rapidly to different environments while they spread in the individual host or even among different hosts (Holland *et al.*,

1982; Holland, 1984, 1993; Steinhauer and Holland, 1987). Errors in DNA replication are infrequent and could be as low as $1x10^{-8}$ to $1x10^{-11}$ per incorporated nucleotide per replication cycle (Smith and Inglis, 1987). This is attributable to the fidelity of DNA dependent DNA polymerases and to DNA proofreading exonucleases that remove misincorporated bases from newly synthesized DNA strands (Holland *et al.*, 1982; Holland, 1984; Smith and Inglis, 1987; Steinhauer and Holland, 1987). In contrast, no proofreading exonuclease function has been found in RNA replication systems and RNA dependent RNA polymerases exhibit low fidelity. Thus RNA base substitution error level per genome is between $1x10^{-3}$ to $1x10^{-4}$ per replication cycle (Steinhauer *et al.*, 1992). The primary source for variation in RNA viruses is point mutation (Smith and Inglis, 1987; Domingo, 1989; Steinhauer *et al.*, 1992). Mutation rates have been measured for several viruses and for different isolates from the same virus. It is important to mention that rate is defined as the number of nucleotide changes in a micro-organism per replication, and substitution rate as the number of mutants reaching fixation per unit time.

RNA virus populations are extremely diverse whenever the number of bases in their genomes exceed or equal the reciprocal of the base substitution error frequency (Domingo, 1989). RNA virus genomes are on average 10 Kb. A virus with a genome of this size and with a base substitution rate of $1x10^{-4}$ will have aproximatelly at least one mutation in most replication cycles (Holland, 1984, 1993; Smith and Inglis, 1987; Steinhauer and Holland, 1987; Steinhauer *et al.*, 1992). Since dengue virus has approximately 10^4 nucleotides, one mutation probably occurs per replication cycle. In general, the probability of mutations in RNA viruses increase with the size of the genome (Steinhauer and Holland,

1987). Molecular evolution as compared with evolution at the phenotypic level has two outstanding features. One is the constancy of the evolution rate for each protein or gene region; the rate of amino acids or nucleotide substitutions is approximately constant per site per year, and is noted as the "molecular evolutionary clock". The other feature is the "conservative nature" of changes; namely, substitutions that are less disruptive to the existing structure or function of the molecule occur more frequently than those changes that are disruptive (Kimura, 1968, 1991; Kimura and Ota, 1971; Gojobori *et al.*, 1990). According to the neutral theory of evolution, mutation pressure plays a predominant role in molecular evolution; however, it tends to be neutral, with neither advantageous nor disadvantageous effects on the phenotype. This is supported by the observation that most of the nucleotide changes in the genome are silent. In RNA viruses, the rate of nucleotide substitutions in the third position of the codon is about twice the rate in the other two positions, resulting in a high frequency of synonymous substitutions. Also in RNA viruses, transitions are often more common than transversions (Holland *et al.*, 1992). The Neutral evolution hypothesis (Kimura, 1968) introduced the important idea of random drift in the occurrence of mutations. The Neutral theory was subsequently modified to address the importance of the mutational pressure as the main cause of molecular evolution and the generation of polymorphism (Ohta, 1974). The new modified theory stresses that the rate of amino acid substitutions in small populations is higher than that in major populations; consequently, substitutions will be concentrated at the time of speciation, when small populations size creates a "bottleneck" (Ohta, 1974).

Muller proposed that in small asexual populations with high mutation rates, genomes free of mutations become rare (Muller, 1964). When the mutation rate is high, the

frequency of mutation-free genomes is reduced. If the populations are small, the mutation-free class can be lost by genetic drift. The loss of mutation-free genomes is irreversible, and the load of deleterious mutations tends to increase. This is followed by the loss of the least-mutated class (Chao, 1992). The least-mutated genomes are eliminated "in a kind of ratchet mechanism" (Muller, 1964). This drift toward sub-populations with more mutations was designated "Muller's Ratchet" (Felsenstein, 1974). Muller's ratchet could be reversed by the occurrence of backward mutations (probably very rare event) or by recombination events. Muller's ratchet theory assumes that the accumulation of highly mutated genomes selects for deleterious mutations (Felsenstein, 1974). In a finite virus population with genetic drift occurring, the mutation-free sub-population becomes very rare and can be lost by chance. Once the mutation free sub-population is lost, the population moves towards a new distribution similar to the previous population, but with a greater number of mutations (Domingo *et al.*, 1976; Chao, 1990, 1991, 1994, 1997; Chao *et al.*, 1997).

Deleterious mutations are independent, therefore their frequencies in a genome do not depend on the presence or abundance of previous deleterious mutations. In this situation the equilibrium mean fitness in an infinite population is equal to e^{-U} (Where U=average number of mutations per genome replication (mutation rate). Taking the average effect of deleterious mutation=(1-S) with $0<S<1$(S is the selective disadvantage caused by a single mutation), a Poisson distribution is obtained, where the frequency of genomes free of mutations C(0) is $e^{-U/S}$. For example, in a RNA virus with a genome of 10^4 nucleotides and a mutation rate of 10^{-4} errors, then $U=10^4/10^{-4}=1$. If S=0.1, the frequency of genomes free of mutations is: $e^{-U/S} = e^{-10} = 4.53\times10^{-5}$ (Chao,

1994). In a finite population subjected to genetic drift, the mutation free sub-population can be lost by chance; those individuals with 0 mutations are very limited and eventually all of them can fail to reproduce in one generation. That sub-population would be lost and there is almost no means to restore it. In that case, Muller's ratchet has advanced one notch. Then the single nucleotide mutation becomes the least mutated sub-population. The resulting population distribution would have a similar shape to the previous distribution, but with a mutation average larger than that of the previous population. In this way, the virus population slowly increases the average number of mutations. It has been suggested that the net result of this process is the decrease of the population fitness, because each new population will have a lower fitness than the previous one (Chao, 1991). Muller's Ratchet and its effects have been demonstrated experimentally, using the RNA bacteriophage φ6 (Chao, 1990). Similar results were reported with the non-segmented RNA phage Q_β (Domingo *et al.*, 1976, 1978). After repetitive passages of the phage, the fitness of the resulting population differed significantly from the parent population. The fitness of the phage population tended to decrease. Isolated clones propagated several times sequentially became as heterogeneous as the parent population but with reduced fitness (Domingo *et al.*, 1976, 1978).

Viral quasispecies

Mutation frequencies in RNA viruses usually range between 10^{-3} to 10^{-6} per site per replication. This is due to the intrinsic error rate of RNA polymerases and also to the lack of proofreading mechanisms. As a consequence, RNA viruses are considered as

population of viral variants. Even infections initiated with a single virion yield a large number of genetic microvariants. These are referred to as a quasispecies (Eigen and Biebricher, 1988). The term quasispecies has been coined to describe a population of closely related molecules that exists in nature in a dynamic process (Eigen and Biebricher, 1988; Eigen *et al.*, 1988; Eigen, 1993a, b). Sub-populations in the quasispecies slowly, but continuously, change. The composition of the quasispecies resulting after a given challenge can be markedly different from the parent quasispecies (Eigen and Biebricher, 1988). The genetic plasticity of the quasispecies can facilitate the rapid adaptation of viruses to new environments.

"Bottlenecks" can modify the quasispecies composition. In nature numerous bottlenecks can affect virus populations. These bottlenecks have two main features: 1) dramatic changes in the environment where viruses are reproducing, and 2) the amplification of new populations from very small groups of individuals.

Virus populations confront a challenge when they replicate in cells from different tissues of the same host during natural infections, or more dramatically, when they infect hosts from diverse species. Virus transmission may result from a very small number of virions. This is known as the "Founder effect" (Carson and Templeton, 1984). The selection of virus sub-populations that result from transmission of HIV or hepatitis C virus from pregnant women to their children (Pang *et al.*, 1991; Wolinsky *et al.*, 1992; Weiner *et al.*, 1993) are examples of founder effects. In these cases, there is a reduced number of viral variants in the newborn compared to those in their respective mothers. Many viruses infect diverse tissues during natural infections and different sub-populations can be found reproducing in different tissues. The microenvironment in the tissue selects those strains

of HIV that successfully grow (more fit) in particular tissues. It has been shown that different sub-populations of HIV predominate in blood and brain tissue (Pang *et al.*, 1991).

Extreme virus-cell confrontations occur in arboviral infections. Arboviruses (arthropod-borne viruses) are transmitted between vertebrate and invertebrate hosts when the vector takes a bloodmeal (Nuttall *et al.*, 1991; Koblet, 1993). The influence of the reproductive microenvironment on virus evolution has been studied with viruses such as vesicular stomatitis virus (VSV) and eastern equine encephalitis virus (Holland *et al.*, 1992; Weaver *et al.*, 1999).

The rate of viral evolution is influenced by the composition of the population in which the virus is reproducing. For VSV, the rate of mutation is higher when serial passages are conducted at a high multiplicity of infection and also in persistent infections (Spindler *et al.*, 1982; de la Torre and Holland, 1990).

In general, fitness tends to decrease during repetitive passages of the virus in cell culture, as predicted by the Muller's Ratchet theory (Holland *et al.*, 1991; Duarte *et al.*, 1992). Using plaque to plaque transfers (genetic bottleneck), accumulation of deleterious mutations occured (Muller's Ratchet effect) resulting in decrease of fitness of mutant clones (Clarke *et al.*, 1993). The intensity of the Muller's Ratchet effect differed among different clones. This suggested that fitness modifications occur randomly. Muller's Ratchet was also be reversed in virus clones that previously experienced its effects (Clarke *et al.*, 1993). This reversal occurred when low-fitness clones were passed repeatedly at high multiplicity of infection. (Clarke *et al.*, 1993).

Viruses often alternate between selective environments in nature. An *in vitro* system composed of BHK or HeLa cells and vesicular stomatitis viruses was used to explore the

effects of these selective environments on the progression of Muller's Ratchet. Progression towards loss of fitness could not be reversed with most of the clones exposed to environmental changes. However, a few clones increased in fitness and this increase was more dramatic when diverse cell lines were used for the passages (Duarte *et al.*, 1993). The average effect of the Muller's Ratchet is to decrease the viral population fitness. In virus-host relations, this effect may favor the survival of the hosts and their progeny, allowing susceptible host populations to support the persistence of viral populations (Duarte *et al.*, 1993).

During passage through natural bottlenecks, virus sub-populations with very reduced fitness could be removed from the general population. The opposite is also possible, resulting in the selection of highly fit populations. A net gain in fitness is reached when sub-populations of similar fitness compete. Although one of the sub-populations finally dominates, both sub-populations increase their fitness (Clarke *et al.*, 1994).

Recently, a population approach was used to study the distribution of fitness levels in VSV. The fitness of many subclones obtained after several plaque to plaque passages was quantified and compared with the distribution of fitness values in the parent population. The distribution of the fitness values in both the parent and mutated populations closely approximated the normal distribution. However, the mutated sub-population had a mean fitness shifted in relation to the mean fitness of the parent sub-population. This result stressed that the evolutionary modification of viral traits follow a stochastic pattern (Duarte *et al.*, 1994). Under certain conditions, the gain of fitness could be dramatic. Serial passages of large virus populations can result in an

exponential increase of fitness (Novella *et al.*, 1995).

Genotype and phenotype selection

Modification in viral genotype and phenotype has been found to occur in several viruses under selective conditions. Specific genomes are selected by exposure of foot and mouth disease virus (FMDV) to polyclonal antibodies in cell culture. The population of virus obtained after serial passages in the presence of antiviral polyclonal antibodies exhibited increased resistance to the polyclonal antibodies, altered electrophoretic mobility and reduction in plaque size (Carrillo *et al.*, 1989; Rojas *et al.*, 1992). Genetic variation has been reported in chronic infections with FMDV. Interestingly, no similar pattern of variation was found in experimentally infected animals (Malirat *et al.*, 1994).

Flaviviruses are very susceptible to change in phenotype under selective conditions. West Nile virus becomes attenuated and loses its ability to infect HeLa cells after only 6 passages (Dunster *et al.*, 1990). Similar results have been found with yellow fever (YF) virus. Monoclonal antibodies that recognize the envelope protein can distinguish between the virus populations before and after the passages (Barrett *et al.*, 1990). Some of the modifications in the phenotype have been associated to the passage of virus on specific cell lines. For example, YF virus becomes temperature sensitive when propagated in C6/36 cell line (Ledger *et al.*, 1992).

Human immunodeficiency virus (HIV) is an excellent example of the concept of quasispecies. Serial passages of the virus in cell culture in the presence of polyclonal antibody selects point mutations in the envelope gene after 4 to 5 weeks (Reitz *et al.*, 1988). Differences in the sub-populations that reside in different tissues were demonstrated; the env gene was different in viruses recovered from blood cells or brain

tissue of HIV patients (Pang *et al.*, 1991). Similar results were found with simian immunodeficiency virus (Burns and Desrosiers, 1991). The heterogeneity of the virus population in monkeys is remarkable; from 27 clones obtained from two infected monkeys, none were similar and all differed from the initial input population.

Drug treatment is another mutant inducer for HIV; differences in virus populations have been detected after short periods of treatment. Nucleotide changes were noted after six months (Boucher *et al.*, 1990), and with more sensitive methods mutations induced by drugs have been detected after a period as short as two weeks after the beginning of the treatment (Wei *et al.*, 1995). HIV can be transmitted from mother to child during pregnancy; however, only certain sub-populations are selectively transmitted. This has been called a "sampling event", and it is a possible selection pressure when viruses are transmitted (Wolinsky *et al.*, 1992; Ganeshan *et al.*, 1997).

During HIV infections, mutations can be selected by the confrontation of the viruses with antibodies. However, many mutants appear very early during primary infections, before antibodies are detected in blood, and the nucleotide sequence changes found in viruses isolated during the acute infection are different from those observed in terminal stages of the disease (Pang *et al.*, 1992). The titer of virus during the infection also has an effect on the rate of mutation. After serial passages, mutations are induced, but the rate is higher when passages are done at low multiplicity of infection (Sanchez-Palomino *et al.*, 1993). This is opposite to that found with VSV (de la Torre and Holland, 1990). All these data point out that the extent of diversity exhibited by HIV in natural or in vitro infections is not due to any fundamental difference in intrinsic biology, but rather to the growth conditions of the virus population. The divergence of HIV to a set of related variants is the

response of a sufficiently large viral population to a variety of subtle selective pressures.

Summary

Dengue is an important cause of morbidity and mortality. Each one of the serotypes of dengue virus includes important genetic diversity in distinct genotypes. Phylogenetic studies have suggested that the increase in the genetic diversity of dengue viruses began about 200 years ago (Zanotto *et al.*, 1996; Holmes, 1998). The introduction of genotypes of dengue-2 virus of Asian origin, associated with the increase in the incidence of DHF in the Americas, suggests the importance of the genetic diversity of dengue viruses and its possible association with the increase in the incidence of more severe clinical cases (Leitmeyer *et al.*, 1999). New molecular approaches can be used now to investigate the level of variability in dengue virus populations and to interpret its significance in terms of disease outcome. In addition, understanding the genetic variability of dengue virus populations and how this is generated will help in the development of new diagnostic and surveillance tools.

In the following chapters, the importance of genetic diversity in dengue virus will be explored. In chapter 2 single strand conformation polymorphism (SSCP) analysis is used to analyze genetic diversity in dengue-2 virus isolates and the sensitivity of the procedure is defined in relation to the number of nucleotide substitutions that can be detected. In chapter 3, SSCP is used to investigate the temporal and spatial genetic diversity of the 4 dengue virus serotypes in the Yucatán Peninsula, México. SSCP allowed the rapid detection of several haplotypes in each one of the 4 serotypes. In Chapter 4 the effect of microenvironment in the generation of genomic diversity in dengue viruses is addressed using RT-PCR, cloning of cDNA, SSCP and sequence information to analyze the

generation of dengue-4 virus mutants by passage of viruses in a cell culture system. Finally in Chapter 5, the association of different sub-populations of dengue-2 viruses with cases of DF and DHF is explored.

Chapter 2

Rapid characterization of genetic diversity among twelve dengue-2 virus isolates by single-strand conformation polymorphism analysis.

INTRODUCTION

A variety of molecular approaches have been used to investigate the molecular epidemiology of dengue viruses (Trent *et al.*, 1983, 1990; Rico-Hesse, 1990; Deubel, 1992; Lanciotti *et al.*, 1992, 1994; Chungue *et al.*, 1993; Morita *et al.*, 1994). Two approaches, RNase T1 oligonucleotide fingerprinting (ONF) and sequence analyses, have proven to be the most revealing in terms of dengue virus population biology. Both techniques have revealed substantial genetic heterogeneity in the respective dengue virus serotypes. The ONF analyses led to the recognition of distinct genetic groups or topotypes (now called genotypes) of dengue viruses, which are associated with distinct geographic regions. Importantly, genetic analysis revealed the origin and movement of viruses between geographic regions. For example, a dengue-2 virus isolated in Trinidad in 1953 originally was designated topotype I (Puerto Rico) and apparently originated on the Indian subcontinent (Rico-Hesse, 1990). This genotype is now the predominant dengue-2 genotype virus in Mexico and Central America (Rico-Hesse, 1990). The dengue-2 virus isolated in Jamaica in 1981 and designated topotype II (Jamaica) was associated with an explosive DHF/DSS (dengue hemorrhagic fever/dengue shock syndrome) outbreak in Cuba the same year (Rico-Hesse, 1990). This strain of dengue-2 virus was subsequently found to have originated in Thailand or Vietnam and is now prevalent in Brazil, Venezuela, and Colombia (Dietz *et al.*, 1990; Rico-Hesse, 1990; Gubler and Trent, 1994).

Clearly, substantial differences exist between topotypes of dengue serotypes. Indeed, even within topotypes, dengue viruses display substantial genotypic variation. Sequence analyses of dengue-2 viruses isolated in Thailand reveal significant genetic variation and demonstrate phenotypic differences in virus interactions with macrophages

(Morens *et al.*, 1991). This may suggest that certain strains are more virulent than others. Similarly, virus strains differ in vector host interactions (Gubler and Rosen, 1977; Rosen *et al.*, 1985; Innis *et al.*, 1988). Thus, virus genetic polymorphisms are well known in nature. The roles that these virus strains play in epidemics, severity of disease outcome, and virus emergence in new areas remain to be determined.

Single-strand conformation polymorphism analysis (SSCP) provides a new method to assess genomic variability among related viral strains. With this procedure it is possible to perform rapid screening of selected fragments of the viral genome to identify regions of genetic variation. Under optimal conditions this technique can identify single base differences. SSCP is based on the differential migration of single strands of DNA (ssDNA) on a non-denaturing polyacrylamide gel (Orita *et al.*, 1989a, b). The double-stranded DNA (dsDNA) obtained from a reverse transcriptase-polymerase chain reaction (RT-PCR) is denatured with heat and/or chemicals and then allowed to renature. Single stranded DNA can renature through intra-strand base pairing to form three-dimensional conformations that are sequence dependent. The newly formed three-dimensional conformations migrate at different rates in the gel, providing a specific banding pattern (haplotype) for each of the different cDNA strands (Orita *et al.*, 1989a, b; Hayashi, 1991). A haplotype is a particular combination of allele, restriction site or any other genetic marker, present in some defined area of the genome. SSCP analyses have been used to demonstrate genomic variation between different viruses and to reveal associations of viral genotypes with specific phenotype (Xi *et al.*, 1993; Enomoto *et al.*, 1994; Black *et al.*, 1995; Katayama *et al.*, 1995; Horie *et al.*, 1996; Oberste *et al.*, 1998). We report here the characterization of genomic

diversity among dengue-2 virus isolates by using SSCP to analyze 3 distinct regions of the viral genome (Farfan *et al.*, 1997).

MATERIAL AND METHODS

Dengue virus isolates

Twelve low-passage isolates of dengue-2 virus strains with known geographic origin, year of isolation, and topotype designation are listed in Table 2.1. The viral specimens were kindly provided by Dr. Duane J. Gubler from the Centers for Disease Control and Prevention (CDC), Division of Vector Borne Infectious Diseases, Fort Collins, Colorado. The nucleotide sequences of the E gene for each of the virus isolates have been published (Deubel *et al.*, 1986; Gruenberg *et al.*, 1988; Blok *et al.*, 1989; Chen and Maguire, 1990; Lewis *et al.*, 1993). The virus isolates were amplified by infecting monolayers of cultured *Ae.albopictus* (C6/36) cells at approximately 0.01 multiplicity of infection and propagated at 28°C in Leibovitz cell culture medium (L-15) containing 10% fetal calf serum, 10%

Sample number	Virus isolate	Topotype	Subtype	Country	Year	Ref.
1	D80-038	Thailand	IIIa	Thailand	1980	(Blok *et al.*, 1989)
2	PUO-218	Thailand	IIIa	Thailand	1980	(Gruenberg *et al.*, 1988)
3	D80-100	Thailand	IIIa	Thailand	1980	(Blok *et al.*, 1989)
4	16681	Thailand	IIIa	Thailand	1964	(Blok *et al.*, 1989)
5	1409	Jamaica	IIIb	Jamaica	1983	(Deubel *et al.*, 1986)
6	D80-141	Thailand	IIIb	Thailand	1980	(Blok *et al.*, 1989)
7	206-714	Sri Lanka	IV	Sri Lanka	1989	(Blok *et al.*, 1989)
8	271-235	Sri Lanka	IV	Sri Lanka	1990	(Blok *et al.*, 1989)
9	1583	Sri Lanka	IV	Sri Lanka	1985	(Blok *et al.*, 1989)
10	1592	Sri Lanka	IV	Sri Lanka	1985	(Blok *et al.*, 1989)
11	#10	Africa	IV	Somalia	1984	(Lewis *et al.*, 1993)
12	Tonga	Americas	V	Tonga	1974	(Chen and Maguire, 1990)

TABLE 2.1 Dengue-2 virus strains used for SSCP analysis.

tryptose phosphate broth, and 100 units penicillin/100 μg streptomycin/ml. Confluent monolayers of C6/36 cell were maintained in L-15 medium containing 2% fetal calf serum. The cultures were incubated for 7-10 days before harvesting.

Reproducibility of SSCP

To assess the reproducibility of SSCP, 19 isolates of dengue-2 from epidemics that occurred in Samoa, Tahiti and Tonga between 1972 and 1974 were analyzed. The strains used were the following: M-32353, M-32859, M-32856, M-32854 from Samoa; 1250, M-32884, M-33370, M-32881 and M-32880 from Tahiti; and S-14620, S-14639, S-14635, S-14730, 1251, S-14707, S-14644, S-14619, S-14694 and S-14616 from Tonga.

RNA purification

C6/36 cells infected with each of the dengue-2 virus strains were harvested by scraping the inner surface of the flask. The cells were pelleted by centrifugation at 1500 rpm for 5 minutes. Total cellular RNA was purified from the infected cells by using the RNeasy kit (Qiagen Inc., Chatsworth, CA), according to the manufacturer's recommendations and then quantified by spectrophotometry.

RT-PCR

Three cDNA fragments derived from the E, NS5 and prM dengue-2 genomic regions were amplified by RT-PCR. RT and PCR amplification were performed sequentially in 0.65 ml Eppendorf tubes in a total reaction of 100 μl. The reaction mixture contained 50 mM KCl, 10mM tris-HCl(pH 9.0), 0.1% Triton X-100, 0.2 mM of each deoxynucleotide triphosphate, 5 mM dithiothreitol, 1.5 mM $MgCl_2$, 50 pmol of each of the primers, 140 U of Superscript II (Life Technologies, Gaithersburg, MD), 3 units of Taq polymerase

(Promega, Madison, WI) and 1-2 µg of sample RNA. For the E cDNA, the reaction was allowed to proceed in a PTC-100 thermocycler (MJ Research, Inc, Waterton, MA) programmed to incubate for 1 h at 42 °C followed by 25 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 51.5 °C for 1 minute and primer extension at 72° C for 2 minutes (Lanciotti *et al.*, 1992). The forward primer (5'-ACATTGGTCACTTTCAAAAAT-3') was identical to nucleotides 1642 to 1662 of dengue-2 Jamaica and the reverse primer (5'-TGGAGAGCCGTCCCCTTCATA-3') was complementary to nucleotides 1912 to 1932. The design of all primers was based on the sequence of the Jamaica strain of dengue-2 (Deubel *et al.*, 1988). The amplified products were analyzed with 1% agarose gels using TAE (0.04 M Tris-acetate, 0.001 M EDTA) as the electrophoresis buffer. To amplify the NS5 and prM fragments, a similar procedure was used, but the annealing temperatures were 53.7 °C and 54.7 °C, respectively. The NS5 forward primer (5'-CAAAAGGAGGACCAGGACACGAAGA-3') sequence was identical to nucleotides 7880 to 7904 and the reverse primer (5'-AATTTTCCACTAAGTTGAGGACTCT-3') was complementary to nucleotides 8056 to 8080. For the prM cDNA, the forward primer (5'-ACCACACGTAACGGAGAACCA-3') was identical to nucleotides 448 to 468 and the reverse primer (5'-TCC CAC ATG TGG AAC GAG TGC-3') was complementary to nucleotides 718 to 738. In both primers there was a single nucleotide difference relative to the sequence in the Jamaica strain. All primers were designed using the Oligo 4.0 software package (National Biosciences, Inc., Plymouth, MN). General parameters proposed by Sharrocks (Sharrocks, 1994) for primer design were followed. The selected primers had a sequence unique within the region to be amplified and low potential for self-annealing.

SSCP

The SSCP procedures used have been described by Orita (Orita *et al.*, 1989a, b) and modified (Black *et al.*, 1995; Marklund *et al.*, 1995). One µl of the PCR product was mixed with 9 µl of denaturing solution (90% formamide, 20 mM NaOH, 0.05% xylene cyanol/ bromophenol blue). The mixture was heated to 95 °C for 3 minutes and then plunged into ice for at least 5 minutes. Two µl of each mixture was loaded directly onto an 8% polyacrylamide gel (37.5:1, acrylamide:bis-acrylamide). Gel dimensions were 20 cm x 20 cm x 0.4 mm. Electrophoresis was performed using a BioRad Protean II apparatus (Bio-Rad Laboratories, Inc. Hercules, CA). Samples were electrophoresed with TBE (53 mM Tris, 53 mM boric acid, 1.5 mM EDTA disodium) buffer at 5 watts for 5 hours at room temperature. Tap water (approximately 22 °C) was passed through the electrophoresis chamber during the entire procedure to prevent overheating. After electrophoresis, the gel was fixed in a 10% acetic acid solution for 20 minutes, and washed three times in distilled water. SSCP banding patterns were then observed by soaking the gel for 30 minutes in a solution containing 0.0058 M silver nitrate and 0.056% formaldehyde. After a 15-second wash in distilled water, the gel was developed at 4°C in a solution of 0.28 M sodium carbonate, 0.056% formaldehyde, and 0.028 mM sodium thiosulfate. The developing process was stopped (by adding an equal volume of 10% acetic acid) when bands were darkly stained and the background was low.

Nucleotide sequencing of E and prM fragments

Primers were designed to amplify a 486 nucleotide fragment in the middle of viral gene that encodes the E protein. This fragment included the 291 nucleotide fragment used

for SSCP analysis. The sequences of the primers used for PCR amplification (1540f and 2005r) are listed on Table 2. Primers also were designed to amplify a 504 nucleotide fragment that included a 291 nucleotide sequence within prM, and used for SSCP analysis. The sequences of these primers (367f and 850r) are also listed in Table 2.2.

Primers for sequencing reactions were designed using the dengue-2 New Guinea C sequence (Irie *et al.*, 1989) and Oligo version 4.0 computer software. Nucleotide sequencing was performed commercially (Macromolecular Resources, Colorado State University) using an ABI 377 DNA Sequencer (Perkin-Elmer Inc., Foster City, CA) with Taq FS polymerase (Perkin-Elmer Inc., Foster City, CA). DNA sequences were analyzed using Seqman™ (Dnastar Inc. Madison, WI) for alignment of the sequence of the two strands and Mega (Kumar *et al.*, 1993) for calculating the nucleotide frequency .

ID	Sequence (5'->3')	Location (5'end)	Fragment size	Annealing temp.
1540f	GAAGACAAAGCTTGGCTGGTG	1540	486	51.6°C
2005r	GCTATCTTTTTCTGTTACGAT			
367f	ATGCTGAACATCTTGAACAGG	367	504	53.9°C
850r	TATGGTGTATGCCAGGATTGC			

Table 2.2 Primers used to sequence fragments of E and prM genes of 12 dengue-2 strains.

RESULTS

RT-PCR amplification

Three cDNAs were amplified and sequenced from each of the 12 dengue-2 virus strains. The observed and predicted size of each cDNA based on the dengue-2 (Jamaica) genomic sequence was 291 bp for the prM and E cDNAs and 201 bp for the NS5 cDNA.

To assess the reproducibility of the RT-PCR and SSCP, we also obtained E cDNA from the 19 strains from the Pacific Islands.

Reproducibility of SSCP

To assess the reproducibility of the procedure, viral strains from Samoa, Tahiti and Tonga were used. The three islands are located in the same geographic area and the viruses were isolated within a period of a few years. Based on these observations, low variability in the viral genomes was expected. SSCP analysis with the 291 nt cDNA from the E locus gave two bands that migrated identically among all the isolates (Figure 2.1).

The nucleotide sequences of the E protein coding gene for the 12 dengue-2 virus strains analyzed with SSCP were available in the Genbank. Using those sequences, the similarities or differences of the SSCP banding patterns were compared with the number of nucleotide differences between every pair of strains.

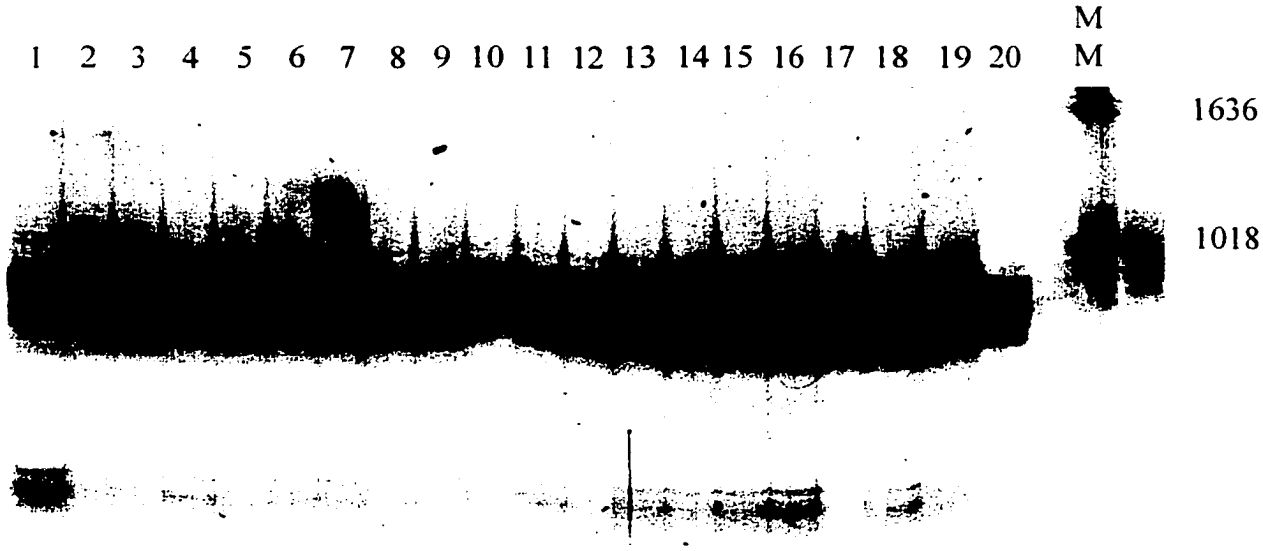

Figure 2.1. SSCP analysis of 291 nt cDNAs generated by RT-PCR from the E gene from viral strains from Samoa, Tahiti, and Tonga. SSCP analysis was performed using the following strains: (1) S-14620, (2) S-14639, (3) S-14635, (4) M-32353, (5) S-14730, (6) 1250, (7) M-32884, (8) 33370, (9) 1251, (10) M32881, (11) M-32859, (12) M-32880 (13) M-32856, (14) M-32854, (15) M-14707, (16) S-14644, (17) S-14619, (18) S-14694 (19) S-14616, and (20) 33370.

Analysis of the E cDNAs by SSCP

The SSCP analysis of the 291 bp cDNA derived from the E gene region demonstrated genotypic diversity among viruses grouped into specific topotypes (Table 2.1 and Figure 2.2 A). Five of the dengue-2 virus isolates (D80-038, PUO-218, D80-100, 16681, and D80-141) have been grouped into genotype III (Thailand) by ONF and sequence analysis (Trent *et al.*, 1983, 1990; Blok *et al.*, 1989). Additional sequence data have grouped these five virus strains into two distinct subtypes designated IIIa (D80-038, PUO-218, D80-100, and 16681) and IIIb (D80-141 virus) (Lewis *et al.*, 1993). Sequence analysis has revealed that the 1409 dengue-2 isolate (Jamaica) is also a IIIb subtype virus (Table 2.1) (Lewis *et al.*, 1993). Of the four viruses classified within subtype IIIa, D80-038, D80-100, and 16681 viruses gave identical SSCP patterns; however, the PUO-218 isolate gave a distinct pattern (Figure 2.2A). E cDNAs from virus isolates D80-100 and 16681 differed by six and seven nucleotide substitutions, respectively, from the cDNA sequence of D80-038 virus (Table 2.3). A sequence comparison of the 291 bp E cDNA of isolates PUO-218 and 16681 revealed that only 4 nucleotide substitutions were needed to form two distinct SSCP patterns. Additionally, the E cDNA sequence of PUO-218 differed by 8 nucleotide substitutions from the D80-038 sequence (Table 2.3), and their SSCP patterns were different. The E cDNA SSCP patterns of IIIb isolates D80-141 and 1409 were clearly distinct and differed by only 5 nucleotides (Table 2.3). The D80-141 and D80-038 cDNA sequences differed by 9 nucleotide substitutions .

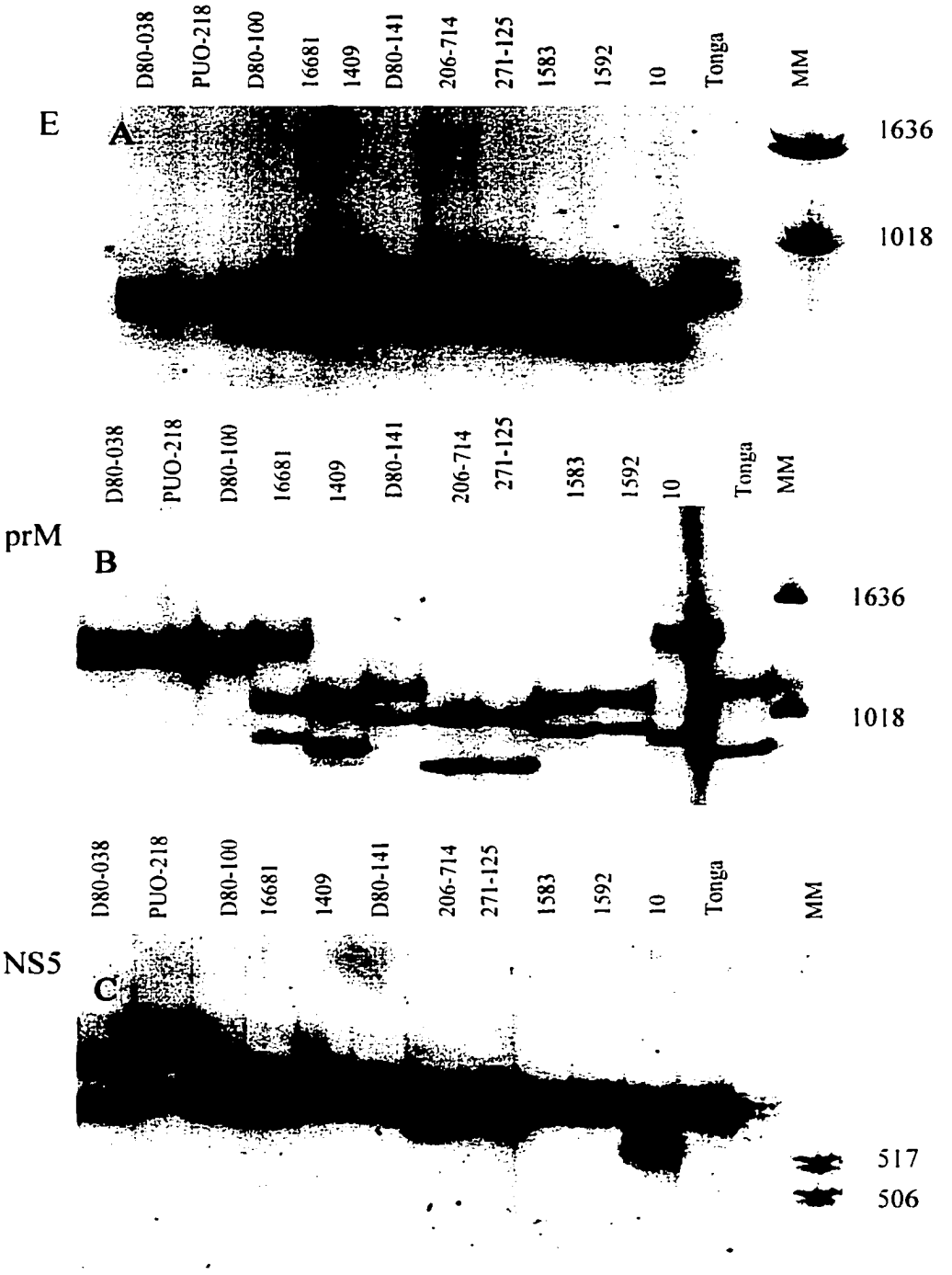

Figure 2.2. SSCP analysis of genetic polymorphisms among 12 dengue-2 virus isolates. SSCP analysis was performed using cDNAs generated by RT-PCR derived from the E, NS5, and prM coding regions of the virus isolates. Shown are SSCP analyses of the 12 virus isolates using (**a**) 291 bp cDNA from E, (**b**) 201 bp cDNA from NS5, and (**c**) 291 bp cDNA from prM coding regions. The 12 dengue-2 virus isolates analyzed were (1) D80-038, (2) PUO-218, (3) D80-100, (4) 16681, (5) 1409, (6) D80-141, (7) 206-714, (8) 271-235, (9) 1583, (10) 1592, (11) #10, and (12) Tonga. Specimens in lines 1-4 are topotype Thailand, subtype IIIa; line 5 topotype Jamaica, subtype IIIb; line 6 topotype thailand, subtype IIIb; lines 7-10 topotype Sri Lanka subtype IV;line 11 topotype Africa, subtype IV and line 12 Topotype Americas, subtype V.

Strain designation	D80-38	PUO-218	D80-100	16681	1409	D80-141	206-714	271-235	1583	1592	10
D80-38	0										
PUO-218	8*,1†,-‡	0									
D80-100	6,1,+	6,0,-	0								
16681	5,1,+	4,0,-	4,0,+	0							
1409	7,2,-	13,1,-	13,1,-	11,1,-	0						
D80-141	6,3,-	11,2,-	11,2,-	9,2,-	4,1,-	0					
206-714	15,2,-	16,1,-	16,1,-	12,1,-	17,0,-	16,1,-	0				
271-235	15,2,-	16,1,-	16,1,-	12,1,-	17,0,-	16,1,-	0,0,+	0			
1583	16,2,-	19,1,-	19,1,-	15,1,-	20,0,-	17,1,-	11,0,-	11,0,-	0		
1592	16,2,-	19,1,-	19,1,-	15,1,-	20,0,-	17,1,-	11,0,-	11,0,-	4,0,+	0	
10	14,3,-	14,2,-	14,2,-	14,2,-	15,1,-	14,2,-	12,1,-	12,1,-	13,1,-	13,1,-	0
Tonga	23,2,-	25,1,-	23,1,-	23,1,-	24,0,-	23,1,-	19,0,-	19,0,-	24,0,-	26,0,-	23,1,-

TABLE 2.3 Number of nucleotide substitutions in the 291 nt sequence of the E gene, among 12 strains of dengue-2 that were analyzed using SSCP

*=transitions †=transversions ‡=band similarity. Band similarity is based on paired comparisons between the bands of each of the strains mentioned on the column header with those corresponding to the strains mentioned on the row header. Two strains are considered similar if SSCP bands migrate the same distance in the electrophoresis gel.

In the four strains of genotype IV (Sri Lanka), two haplotypes were differentiated. Identical SSCP patterns and sequences of the 291 bp region of E were observed for viruses 206-714 and 271-235 that were isolated in 1989 and 1990, respectively. Identical SSCP patterns also were observed for genotype IV viruses 1583 and 1592, both of which were isolated in 1985 (Figure 2.2A). The cDNAs from these viruses differed by 4 nucleotide substitutions. The cDNAs of 206-714 and 271-235 viruses differed from the 1583 and 1592 viruses by 11 nucleotide substitutions in this region of the E gene (Table 2.3). The isolate from topotype IV (Africa) had a banding pattern distinct from either 206-714 and 271-235 or 1583 and 1592 viruses (Figure 2.2A). This virus contained 13 nucleotide changes from both 206-714 or 271-235 virus sequences and 14 nucleotide changes from

both 1583 or 1592 virus sequences (Table 2.3). Finally, the Tonga isolate from genotype V (Americas) had a unique SSCP pattern. The 291 bp E cDNA differed by 19 and 26 nucleotide substitutions from the other eleven dengue-2 virus isolates (Table 2.3).

Sensitivity of SSCP for detecting identical sequences was 100%. Two identical sequences resulted in identical SSCP banding patterns (samples 206-714 and 271-235), but specificity was only 50%. Five sequences that were different, produced shared patterns (samples D80-38,D80-100, 16681 and 1592). Positive and negative predictive values were 26% and 100%, respectively.

SSCP analysis and nucleotide sequencing of the E cDNAs

In order to verify the observed differences in SSCP haplotypes and the number of nucleotide replacements, the 291 nucleotide fragments of the E gene of the 12 strains analyzed by SSCP were sequenced. The number of pairwise nucleotide substitutions between each pair of strains is presented in Table 2.4, and the 291 nucleotide sequences of the 12 strains are presented in Figure 2.3.

The number of nucleotide differences among strains were compared using Genbank sequences and the sequences determined at CSU. When the Genbank sequences were used, in 25 out of 66 pairs the number of nucleotide differences was higher than that obtained using the CSU sequences. In 9 out of 66 pairs, the number of differences was lower, and in the remaining 32 pairs, there were no differences between the results obtained using Genbank or CSU sequences.

Strain designation	D80-038	PUO-218	D80-100	16681	1409	D80-141	206-714	271-235	1583	1592	10
D80-38	0										
PUO-218	6*,0†,-‡										
D80-100	2,0,+	6,0,-									
16681	4,0,+	4,0,-	4,0,+	0							
1409	13,1,-	13,1,-	13,1,-	11,1,-	0						
D80-141	13,1,-	13,1,-	13,1,-	11,1,-	3,1,-	0					
206-714	14,1,-	16,1,-	16,1,-	12,1,-	17,0,-	18,0,-	0				
271-235	14,1,-	16,1,-	16,1,-	12,1,-	17,0,-	18,0,-	0,0,+	0			
1583	17,1,-	19,1,-	19,1,-	15,1,-	20,0,-	19,0,-	11,0,-	11,0,-	0		
1592	16,1,-	18,1,-	18,1,-	14,1,-	19,0,-	18,0,-	10,0,-	10,0,-	3,0,+	0	
10	14,1,-	14,1,-	14,2,-	14,1,-	15,0,-	16,0,-	12,0,-	12,0,-	13,0,-	12,0,-	0
Tonga	23,1,-	25,1,-	23,1,-	23,1,-	24,0,-	25,0,-	19,0,-	19,0,-	24,0,-	25,0,-	23,0,-

*=transitions †=transversions ‡=band similarity. Band similarity is based on paired comparisons between the bands of each of the strains mentioned on the column header with those corresponding to the strains mentioned on the row header. Two strains are considered similar if SSCP bands migrate the same distance in the electrophoresis gel.

TABLE 2.4 Number of nucleotide substitutions in the 291 nt sequence of the E gene, among 12 strains of dengue-2 that were analyzed using SSCP

Using the actual sequences, the four strains in subtype IIIa (D80-038, PUO-218, D80-100 and 16681) differed by no more than 6 nucleotides (Table 2.4). When the Genbank sequences were compared, the number of nucleotide substitutions was greater (Table 2.3). The two strains in subtype IIIb [1409 (Jamaica) and D80-141] differed from the three subtype IIIa strains (D80-038, PUO-218 and D80-100) by 14 nucleotides and from the other strain in the subtype IIIa (16681) by 12 nucleotides. It is important to note that the first three IIIa subtype strains were isolated from cases in 1980, and the last one from a case in 1964. The number of nucleotide differences between the two strains in subtype IIIb was 4 using the CSU sequences and 5 using the Genbank sequences. Their SSCP banding patterns were clearly different. The two pairs of strains from Sri Lanka gave identical SSCP banding pattern. The sequences of the first pair of strains (206-714 and

271-235) from Sri Lanka were identical. There were 3 nucleotide differences between the other two strains (1583 and 1592). The Tonga strain had the highest number of nucleotide replacements as compared with all the other strains.

SSCP analysis and nucleotide sequencing of the prM cDNAs

Three unique SSCP haplotypes were identified from the 291 bp prM cDNAs derived from the subtype IIIa dengue-2 viruses (Figure 2.2B). Virus isolates D80-038 and PUO-218 displayed identical SSCP patterns that were distinct from the unique SSCP patterns of virus isolates D80-100 and 16681 (Figure 2.2B). However, D80-038 and PUO-218 differed by two substitutions (Table 2.4). The prM 291 nucleotide sequence of the twelve strains are presented in Figure 2.4. The two viruses from subtype IIIb (D80-141 and 1409) each had unique SSCP patterns. In addition, they differed from the other ten dengue-2 viruses analyzed. D80-038 and D80-100 differed in a single substitution, but the banding patterns were clearly different. The four genotype IV (Sri Lanka) isolates displayed three distinct SSCP patterns. Isolates 206-714 and 271-235 gave identical SSCP patterns, consistent with their E and NS5 SSCP patterns (Figure 2.2B). Nucleotide sequences of these two strains were identical. Sri Lanka dengue-2 isolates 1583 and 1592 differed in their prM cDNA SSCP patterns, and the nucleotide sequencing revealed 2 substitutions. When the E protein coding gene of these two strains were compared, the SSCP banding patterns were similar even though three nucleotide substitutions existed. The two isolates #10 and Tonga [genotype IV (Africa) and V (Americas), respectively] also had unique SSCP patterns that were distinct from the other 10 dengue-2 viruses (Figure 2.2B).

SSCP analysis of the NS5 cDNAs

The 201 bp cDNAs derived from the NS5 gene of the twelve dengue-2 virus isolates were analyzed by SSCP. Three subtype IIIa isolates (D80-038, D80-100, and 16681) had identical SSCP patterns, which was also seen with SSCP analysis of the E cDNAs (Figure 2.2C). The cDNA from isolate PUO-218 displayed a SSCP pattern distinct from the other IIIa dengue-2 viruses. Interestingly, the NS-5 201 SSCP pattern of one subtype IIIb isolate, 1409 (Jamaica) was identical to the patterns of the three subtype IIIa isolates, D80-038, D80-100 and 16681. The D80-141 isolate was distinct from the other genotype III viruses (Figure 2.2C). The four genotype IV (Sri Lanka) isolates displayed two distinct SSCP patterns, which was consistent with the groupings found with SSCP analysis of the E cDNAs.

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Nt.              1 1111111112 2222222223 3333333334 4444444445 5555555556
position 1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

 D80-038 ACATTGGTCA CTTTCAAAAA TCCCCATGCG AAGAAACAGG ATGTTGTTGT TTTAGGATCC
 PUO-218 .......... .......... ...T...... .......... .......... ..........
 D80-100 .......... .......... .......... .......... .......... C.........
 16681   .......... .......... .......... .......... .......... ..........
 1409    .......... .......... ......C... ........A. ....C..... .........T
 D80-141 .......... .......... ......C..A .......... ....C..... .........T
 206-714 .......... .C........ .......... ..A....... .......... ..........
 271-235 .......... .C........ .......... ..A....... .......... ..........
 1583    ...C...... .......G.. .........A .......... .......... ..........
 1592    .......... .......... .........A .......... .......... ..........
 10      .......... .......... ...T...... .......... .......... C.........
 tonga   ...C...... .C........ .......... ..A....... .......... C.........


                                                    1 1111111111 1111111111
         6666666667 7777777778 8888888889 9999999990 0000000001 1111111112
         1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

D80-038  CAAGAAGGGG CCATGCATAC AGCACTCACA GGAGCCACAG AAATCCAAAT GTCATCAGGA
PUO-218  .......... .......C.. .......... ..G....... .......... ..........
D80-100  .......... .......C.. .......... .......... .......... ..........
16681    .......... .......C.. ......T... ..G....... .......... ..........
1409     .......... .......C.. G......... ..G....... .......G.. ..........
D80-141  .......... .......C.. G......... ..G....... .......G.. ..........
206-714  .......... .T........ ......T... ..G.....G. .......G.. ..........
271-235  .......... .T........ ......T... ..G.....G. .......G.. ..........
1583     .......... .T........ ......T... ..G.....G. .......G.. ..........
1592     .......... .T........ ......T... ..G.....G. .......G.. ..........
10       .......... .T........ .......... ..G.....G. .......G.. ..........
tonga    .....G.... .......... .......... ..G..T..G. .......G.. ..........


         1111111111 1111111111 1111111111 1111111111 1111111111 1111111111
         2222222223 3333333334 4444444445 5555555556 6666666667 7777777778
         1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

D80-038  AACTTACTCT TCACAGGACA TCTCAAGTGC AGGCTGAGAA TGGACAAGCT ACAGCTCAAA
PUO-218  .......... .......... .......... .......... .......... ..........
D80-100  .......... .......... .......... .......... .......... ..........
16681    .......... .......... .......... .......... .......... ..........
1409     ........G. .......... .......... .......... .......A.. ..........
D80-141  ........A. .......... .......... .......... .......A.. ..........
206-714  ...C....G. .......... .......... .......... .......A.. ..........
271-235  ...C....G. .......... .......... .......... .......A.. ..........
1583     ...C....G. .......... C......... .......... .......A.. ..........
1592     ...C....G. .......... C......... .......... .......A.. ..........
10       ........G. .......... C......... .......... .......A.. ..........
tonga    ...C.G..G. .......... .......... .......... .......AT. ...A..T...
```

Figure 2.3. Nucleotide sequence of a 291 nt fragment of the gene that codes for E protein of 12 strains of dengue-2 virus. Sequence was provided by Macromolecular Resources at CSU.

```
          1111111111 1111111112 2222222222 2222222222 2222222222 2222222222
          8888888889 9999999990 0000000001 1111111112 2222222223 3333333334
          1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

D80-038   GGAATGTCAT ACTCTATGTG CACAGGAAAG TTTAAAGTTG TGAAGGAAAT AGCAGAAACA
PUO-218   .......... .......... .......... .......... .......... ..........
D80-100   .......... .......... .......... .......... .......... ..........
16681     .......... .......... .......... .......... .......... ..........
1409      .......... .......... T......... ......A... .......... ..........
D80-141   .......... .......... T......... ......A... .......... ..........
206-714   .......... .......... .......... .......... .A........ ..........
271-235   .......... .......... .......... .......... .A........ ..........
1583      ..G....... .......... T......... .....G.... .......... ..........
1592      ..G....... .......... T......... .....G.... .......... ..........
10        .......... .T........ T......... .......... .......... ..........
tonga     ..G....... ....C..... .......... ......A... .......... ..........


          2222222222 2222222222 2222222222 2222222222 2222222222 2
          4444444445 5555555556 6666666667 7777777778 8888888889 9
          1234567890 1234567890 1234567890 1234567890 1234567890 1

D80-038   CAACATGGAA CGATAGTTAT CAGAGTGCAA TATGAAGGGG ACGGCTCTCC A
PUO-218   .......... .A........ ...G.....G .......... .......... .
D80-100   .......... .......... .......... .......... .......... .
16681     .......... .A........ .......... .......... .......... .
1409      .......... .A........ ......A... .......... .......... .
D80-141   .......... .A........ ......A... .......... .......... .
206-714   .......... .A........ ......A... .......... .T..T..... .
271-235   .......... .A........ ......A... .......... .T..T..... .
1583      .......... .A........ .......... .......... .T..T..... .
1592      .....C.... .A........ .......... .......... .T..T..... .
10        .......... .A......G. ......A... .......... ....T..... .
tonga     .......... .A.....C.. T.....A... ........A. .......... .
```

Figure 2.3 (Continued).

```
Nt.                1 1111111112 2222222223 3333333334 4444444445 5555555556
Posi     1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
tion
D80-038  ACCACACGTA ACGGAGAACC ACACATGATC GTCAGTAGAC AAGAGAAAGG GAAAAGTCTT
PUO-218  .......... .......... .......... .......... .......... ..........
D80-100  .......... .......... .......... .......... .......... ..........
16681    .......... .......... .......... .....C.... .......... ..........
1409     .......... .T........ .......... ..TG....G. .......... ..........
D80-141  .......... .T........ .......... ..TG...... .......... ..........
206-714  ........C. .......... .......... .....C.... .......... .........C
271-235  ........C. .......... .......... .....C.... .......... .........C
1583     ........C. .......... .......... .......... .......... .........C
1592     ........C. .......... .......... .......... .......... .........C
10       ........C. .......... .......... .......... .......... ..........
TONGA    ........C. .......... .........T ........G. ....A..... ......C...


                                                     1 1111111111 1111111111
         6666666667 7777777778 8888888889 9999999990 0000000001 1111111112
         1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
D80-038  CTGTTTAAAA CAGAGGATGG CGTGAACATG TGCACCCTCA TGGCCATGGA CCTTGGTGAA
PUO-218  T......... .......... .......... .......... .......... ..........
D80-100  .......... .......... .......... .......... .......... ..........
16681    .......... .......... .......... ..T....... .......... ..........
1409     .....C.... .......... T..T...... ..T....... .......A.. T.........
D80-141  .....C.... .......... T......... ..T....... .......A.. T.........
206-714  T......... .......... T......... ..T....... .......... ..........
271-235  T......... .......... T......... ..T....... .......... ..........
1583     .....C.... .......... TT........ ..T....... .......... ..........
1592     .....C.... .......... TT........ ..T....... .......... ..........
10       .......... .......... T......... ..T....... .......... T.........
TONGA    .....C..G. ..A....... .AC....... ..T....... .......... .........G


         1111111111 1111111111 1111111111 1111111111 1111111111 1111111111
         2222222223 3333333334 4444444445 5555555556 6666666667 7777777778
         1234567890 1234567890 1234567890 1234567890 1234567890 1234567890

D80-038  TTGTGTGAAG ACACAATCAC GTACAAGTGT CCCCTTCTCA GGCAGAATGA GCCAGAAGAC
PUO-218  .......... .......... .......... .......... .......... ..........
D80-100  .......... .......... .......... .......... .......... ..........
16681    .......... .......... .......... .......... .......... ..........
1409     .......... .T........ .......... .....C.... ....A..... A.........
D80-141  .......... .T........ .........C .....C.... .......... A.........
206-714  C......... .......... T..T..C... ..T....... .......... A........T
271-235  C......... .......... T..T..C... ..T....... .......... A........T
1583     .......... .......... T..T..C... ..T....... .......... A........T
1592     .......... .......... T..T..C... ..T....... .......... A........T
10       .......... .......... T..T..T... ..T....... .......... A.........
TONGA    .......... .......... ...T..A... ..TT...... A......C.. A.........
```

Figure 2.4. Nucleotide sequence of a 291 nt fragment of the gene that codes for prM protein of 12 strains of dengue-2 virus. Sequence was provided by Macromolecular Resources at CSU.

```
         1111111111 1111111112 2222222222 2222222222 2222222222 2222222222
         8888888889 9999999990 0000000001 1111111112 2222222223 3333333334
         1234567890 1234567890 1234567890 1234567890 1234567890 1234567890
D80-038  ATAGACTGTT GGTGCAACTC CACGTCCACG TGGGTAACCT ATGGGACTTG TACCACCACG
PUO-218  ........C. .......... .......... .......... .......... ..........
D80-100  .......... .......... .......... .......... .......... ...T......
16681    .......... .......... T......... ........T. .......G.. .........T.
1409     .....T.... .......... T........A ........T. .......A.. .G.......A
D80-141  .....T.... .......... T........A ........T. .......G.. ..........A
206-714  .....T.... .......... .........A ........T. .......A.. C..TG....A
271-235  .....T.... .......... .........A ........T. .......A.. C..TG....A
1583     .......... .......... .........A ........T. .......A.. C..GG....A
1592     .....T.... .......... .........A ........T. .......A.. C..TG....A
10       .......... .......... .........A ........T. .......A.. C...G....A
TONGA    .....T.... .......... .........A ........T. .......A.. ..........A

         2222222222 2222222222 2222222222 2222222222 2222222222 2
         4444444445 5555555556 6666666667 7777777778 8888888889 9
         1234567890 1234567890 1234567890 1234567890 1234567890 1
D80-038  GGAGAACATA GAAGAGAAAA AAGATCAGTG GCACTCGTTC CACATGTGGG A
PUO-218  .......... .......... .......... .......... .......... .
D80-100  .......... .......... .......... .......... .......... .
16681    .......... .......... .......... .......... .......... .
1409     ........C. .......... .......... .......... .......... T
D80-141  ........C. .......... .......... .......... .......... T
206-714  ........C. ....G..... .......... ........C. .......... .
271-235  ........C. ....G..... .......... ........C. .......... .
1583     ........C. ....G..... .......... ........C. .......... .
1592     ........C. ....G..... .......... ........C. .......... .
10       ........C. ....G..... .......... .......... .......... .
TONGA    .....G.GC. .......... .......... ..G..T.... ....C..... .
```

Figure 2.4. (Continued).

Strain designation	D80-038	PUO-218	D80-100	16681	1409	D80-141	206-714	271-235	1583	1592	10
D80-38	0										
PUO-218	2*,0†,+‡										
D80-100	1,0,-	3,0,-	0								
16681	5,1,-	7,1,-	6,1,-	0							
1409	20,3,-	22,3,-	21,3,-	20,2,-	0						
D80-141	18,2,-	20,2,-	19,2,-	17,1,-	5,1,-	0					
206-714	21,3,-	21,3,-	20,3,-	21,2,-	25,4,-	24,3,-	0				
271-235	21,3,-	21,3,-	20,3,-	21,2,-	25,4,-	24,3,-	0,0,+	0			
1583	17,5,-	19,5,-	17,5,-	19,4,-	21,6,-	20,5,-	5,2,-	5,2,-	0		
1592	19,4,-	21,4,-	18,4,-	21,3,-	21,5,-	20,4,-	4,1,-	4,1,-	1,1,-	0	
10	14,3,-	16,3,-	15,3,-	16,2,-	18,4,-	17,3,-	10,0,-	10,0,-	6,2,-	8,1,-	0
Tonga	29,1,-	31,1,-	30,1,-	31,0,-	31,2,-	32,1,-	29,2,-	29,2,-	24,4,-	24,3,-	24,2,-

*=transitions †=transversions ‡=band similarity; Band similarity is based on paired comparisons between the bands of each of the strains mentioned on the column header with those corresponding to the strains mentioned on the row header. Two strains are considered similar if SSCP bands migrate the same distance in the electrophoresis gel.

TABLE 2.5 Number of nucleotide substitutions in the 291 nt sequence of the prM gene, among 12 strains of dengue-2 that were analyzed using SSCP

DISCUSSION

SSCP analysis was originally developed to detect point mutations between two DNA sequences by comparing the migration rates of renatured ssDNA molecules on nondenaturing polyacrylamide gels (Orita *et al.*, 1989a, b). These studies revealed that SSCP analysis could detect approximately 99% of the point mutations when the DNA molecule was less than 300 bp in length (Hayashi, 1991). This technique demonstrated that the formation of stable intrastrand interactions was highly sensitive to the primary sequence of the DNA molecule. Banding patterns obtained with SSCP of dengue virus sequences were highly reproducible (Figure 2.1).

The SSCP patterns of the E and NS5 cDNAs from the 12 dengue virus isolates showed identical groupings within genotypes, with two distinct genotypes of viruses in topotype IIIa, two genotypes in topotype IIIb, and two genotypes in topotype IV. The SSCP patterns of prM cDNAs suggested that a greater number of nucleotide substitutions occurred in this region of the genome because three distinct genotypes were identified in topotypes IIIa and IV.

When this research was initially conducted, nucleotide sequences available in Genbank were used to correlate the banding patterns obtained by SSCP and the nucleotide sequences of the 12 dengue-2 virus strains. To confirm the conclusions, the E and prM fragments were sequenced. Once these sequences were available, a re-evaluation of the number of nucleotide differences that SSCP could identify was conducted. The analysis of the 291 nucleotide fragment of the E gene revealed that the threshold for producing different SSCP banding patterns was a 4 nucleotide difference.

SSCP analysis of the 291 bp E cDNA revealed that two virus strains (PUO-218 and 16681), which differed by 4 nucleotides, yielded distinct SSCP patterns. All of the cDNAs giving identical SSCP patterns had 4 or less nucleotide substitutions. Isolates 1583 and 1592 had identical SSCP patterns but differed by 3 nucleotides. Isolate D80-141 differed from 1409 by 4 nucleotide substitutions and had unique SSCP banding patterns. In general, two cDNAs that differed by greater than 4 nucleotide substitutions gave distinct SSCP patterns.

Strains D80-038 and 16681 differed by 4 nucleotides. The same number of differences was found between strains D80-100 and 16681. In both cases, the banding patterns between each pair of strains were identical. However, pairs 1409 and D80-141 and PUO-218 and 16681 each differed in 4 nucleotides, but their banding patterns were clearly different. The nucleotide sequences of two additional pairs differed by fewer than 4 nucleotides and had identical SSCP banding patterns. (Strains D80-038 and D80-100 differed in by 2 nucleotides and 1583 and 1592 by three nucleotides). Strains 206-714 and 271-235 had identical sequences. Their SSCP banding patterns were identical. Two pairs of strains (D80-038 and PUO-218, and PUO-218 and D80-100) differed in 6 nucleotides; both of these pairs yielded different banding patterns. Pairwise comparisons of the nucleotide sequences of the remaining strains, revealed more than 6 nucleotide differences between the members of the pairs. The SSCP banding patterns were different in all the cases. The maximum number of nucleotide substitutions between sequences of paired strains in the same topotype and subtype was 6 (strains D80-038, PUO-218, D80-100 and 16681, topotype Thailand, subtype IIIa).

Sequence analysis of the prM gene revealed that the threshold of nucleotide differences required to produce different SSCP banding patterns was 2. Strains D80-038 and PUO-218 differed by only 2 nucleotides, and their SSCP banding patterns were identical. However, dengue-2 strains 1583 and 1592 also differed by 2 nucleotides and produced different banding patterns. Two strains with identical sequences, 206-714 and 271-235 produced identical banding patterns. As an exception to the 2 nucleotide threshold, strains D80-038 and D80-100 had only one nucleotide difference and their banding patterns were clearly distinct. All the other paired comparisons among the strains differed by more than 2 nucleotides, and in all the cases the SSCP banding patterns were also different.

A comparison of the 291 nucleotide sequences of the 12 dengue-2 strains revealed other important differences. Pairwise comparisons of the nucleotide sequences of the E and prM genes revealed that the total number of transitions for E and prM were 961 and 1146, respectively. The total number of transversions also was different with 32 for E and 159 for prM. It was noteworthy that for the E gene, of the 32 transversions found in the paired comparison of the sequences, 28 (87.5%) were G↔C and only 4 (12.5%) were A↔C. In contrast for prM, the four possible transversions were present. From a total of 159 transversions, 44(27.6%) were A↔T, 4 (2.5%) A↔C, 80 (50.3%) T↔G and 31(19.5%) C↔G.

The sequencing of the genomic fragments provided evidence indicating that the threshold of nucleotide differences yielding differences in the SSCP banding patterns is lower than that estimated based on the sequences in the Genbank. These differences are

most likely explained by the different passage history of the viruses when they were sequenced.

ONF was an important step in identifying dengue virus genotypes and was based on the digestion of viral RNA with ribonuclease T_1, which cleaves after guanosine residues (Kew and Nottay, 1986). Only the large T1 RNase oligonucleotides (10 to 40 nt) from each virus isolate could be compared after 2-dimensional electrophoresis on a polyacrylamide gel. It is estimated that these oligonucleotides represented 10% to 15% of the viral genome (Repik *et al.*, 1983). Additionally, it has been estimated that ONF patterns showing 25% and 85% identity indicate from 90% to 99% base sequence similarity, respectively (Repik *et al.*, 1983). Given that the complete viral genome is digested, the homologies found through fingerprinting represent an average of the similarities in the genomes under study.

In contrast, SSCP is based on differential mobilities among three-dimensional conformations of ssDNA in polyacrylamide gel, arising from difference in their nucleotide sequences. In this case, the genomic location of the fragment to be analyzed is decided in advance, and identical SSCP banding patterns correspond to closely related cDNA sequences. However, it is important to mention that the location where the nucleotide difference among the cDNAs been compared, has effect on the sensitivity of the analysis. Nucleotide differences located in the ends of ssDNAs, do not result in significant structural changes in the tri-dimensional conformations. SSCP analysis can be used to screen selected regions in the genome through the use of different sets of primers. The SSCP procedure presented here did not require the use of radioactivity and used equipment

frequently present in research and diagnostic laboratories. Complete SSCP analysis, beginning with the viral isolates and ending with the stained gel, can be done in one day. SSCP is a sensitive and easy procedure for fast screening of dengue virus genomic variants. Nucleotide sequencing is the gold standard when comparing virus isolates. However SSCP is an excellent procedure for approach to identify rapidly differences in nucleotide sequences and is faster and cheaper than sequencing.

CHAPTER 3

SSCP ANALYSIS OF DENGUE VIRUS GENOMES FROM YUCATÁN PENÍNSULA IN MÉXICO

INTRODUCTION

From 1940's to 1960's health authorities in México conducted intensive campaigns to reduce the density of the mosquito *Aedes aegypti*. México along with many other countries in Latin America participated in the *Ae. aegypti* eradication campaign. As a result the mosquito was officially declared eradicated from the country in 1963, after verification by the Pan American Health Organization (Secretaría de Salud, 1992). However, a few countries did not participate in the eradication campaign (Secretaría de Salud, 1992). A surveillance system was therefore established to monitor for reinfestation of the countries that were *Ae. aegypti* free. In spite of the effort and money invested in the campaign, *Ae. aegypti* was detected in the northern region of México in 1965 only two years after the eradication. The southern border also was infested by 1975.

The first cases of dengue in México were identified in 1978 in Tapachula, Chiapas (Secretaría de Salud, 1992). During 1979 a total of 6,187 cases of dengue were reported. Nine of the 32 states in México reported dengue that year. In 1980, fifteen states reported cases. By 1981, dengue was recognized in the states of the Pacific coast of México and in the northern state of Sinaloa. A total of 17,046 cases were reported that year. The first serotype of dengue identified in México in 1980, was dengue-1 from a patient from Mérida, Yucatán (Anonymous, 1980). Dengue-1 and dengue-2 were isolated in 1982 in other regions of the country and by 1983, dengue-4 had also been isolated.

During 1984 and 1985, dengue was reported in 25 states. During the following years, dengue-1, dengue-2 and dengue-4 were reported throughout México. During 1995, dengue-3 was isolated for the first time in México (Briseño-García *et al.*, 1996), and by

September of that year, the first isolation of dengue-3 virus was obtained from a patient in Yucatán.

Dengue in the Yucatán State

In the Yucatán State thousands of cases of dengue were reported each year from 1979 to 1981. The virus serotype that circulated was dengue-1. During 1984, dengue serotype 4 was introduced, causing a major epidemic with more than 5,000 reported cases. During that year most of the viral isolates were dengue-4 and only one was dengue-1 (Loroño-Pino *et al.*, 1993). Several cases of dengue fever with hemorrhagic manifestations were reported. Nine patients presented with severe hemorrhagic manifestations and four of them died. However, only one met the WHO criteria of DHF. During the 1984 epidemic, the disease was confirmed by serology and/or virus isolation in 200 patients. In another 4,863 patients, the diagnosis was based on clinical signs and symptoms. The following years, only laboratory confirmed infections were included in the reporting system.

The Yucatán is considered an endemic area of dengue, since virus transmission occurs each year. In a serologic study conducted in the state during 1985, 72.5% of the people tested had antibodies against dengue viruses (Loroño *et al.*, 1989). In 1991, dengue-2 was introduced in the region, and 364 cases were confirmed by serology or virus isolation. During the next years the number of cases decreased considerably, and in 1994 another 531 cases were confirmed. Twenty five dengue viruses were isolated at this time. Twenty one of the isolates were dengue-1 (84%). Three isolates were dengue-2 and one was dengue-4. Dengue-1 was isolated in 1980, and it was considered the serotype

responsible for endemic transmission in Yucatán. The large number of cases that occurred during the 1994 epidemic and the fact that dengue-1 was the predominant serotype isolated that year, suggested the possibility that the viruses that circulated in the 1994 epidemic differed antigenically from the dengue-1 that circulated in previous years. Thus, the human population was susceptible to this viral variant. In 1995, another dengue epidemic occurred and 58 cases were confirmed in the laboratory. Fourteen virus isolates were obtained; nine of them (64%) were dengue-4, four (28%) dengue-1 and one (7%) dengue-3. This was the first isolate of dengue-3 in the Yucatán Península. In 1996, 414 cases were confirmed by laboratory analyses. Of 44 isolates, 35 (79.5%) were dengue-3. Dengue-1, dengue-2 and dengue-4 viruses also were isolated. In 1997, 4,750 cases of dengue were reported, 1722 were confirmed in the laboratory, and an additional 3,028 met the clinical definition of dengue, but no serological diagnosis or virus isolation was attempted. Virus isolation was attempted with only a fraction of the serum samples collected that year. The predominant serotype was dengue-3 (92% of the specimens), but dengue-1 and 4 also were isolated. A total of 195 cases met the diagnosis of DHF and 10 fulfilled the criteria for the severest disease form, dengue shock syndrome (DSS).

Previously, we have used single-strand conformation polymorphism (SSCP) analysis to successfully characterize strains of dengue-2 (Farfan *et al.*, 1997). The SSCP patterns and nucleotide sequences of a 291 nt fragment of the prM region of dengue-2 virus genomes were compared. Nucleotide sequences that were identical, produced identical SSCP haplotypes. Two dengue-2 viruses with sequences that differed by only 2 nucleotides (positions 61 and 189), gave identical SSCP haplotypes. However, two

sequences that differed in only one nucleotide (position 234), produced different SSCP haplotypes. A detailed analysis of these results including the number of nucleotide substitutions and the SSCP banding patterns associated with them was presented in Chapter 2. SSCP would provide a rapid and sensitive technique for identifying the various haplotypes that have circulated in the Yucatan and would allow rapid surveillance for the introduction of new haplotypes in the region. Dengue isolates have been collected from epidemics in Yucatan since 1981.

Studies were conducted to determine the extent of genetic variability in the dengue viruses that have circulated in the Yucatan peninsula. The hypothesis for this research was that RT-PCR and SSCP analysis could be used to characterize genetic diversity in dengue virus serotypes in the Yucatan.

Material and Methods

Source of the viruses

In 1983 serological studies and virus isolation for dengue diagnosis were initiated at the Centro de Investigaciones Regionales "Dr. Hideyo Noguchi" of the Universidad Autónoma de Yucatán. The Center routinely analyzes serum for dengue viruses antibodies by hemagglutination inhibition (HI) and IgM enzyme linked immunosorbent assay (IgM-ELISA). Virus isolation is also performed. However during periods when virus isolation is not possible, the sera are carefully stored at -70°C. For the isolation, storage, and analysis of the viruses, we have collaborated with the Division of Vector-Borne Diseases, Centers for Disease Control and Prevention (CDC), and in more recent years also with the Arthropod-Borne and Infectious Diseases Laboratory (AIDL) from the Department of

Microbiology in Colorado State University; both Institutions are located in Fort Collins, Colorado, USA.

Most of the viruses characterized in the present study were obtained from serum samples from patients with suspected dengue disease. All the patients were residents of the Yucatán Península, most of them from the Yucatán State (Figure 3.1). Dengue virus isolates were obtained from 22 of the 106 municipalities from the state of Yucatán. In addition, some viral isolates were obtained from patients from Campeche, Quintana Roo and Cozumel in the Yucatán Peninsula, and from the State of Jalisco (Figure 3.1). Seventy four (51%) of the viral isolates were from patients from Mérida, 51 (36%) were from other municipalities in Yucatán and 7 samples (5%) were from patients from the other states in the Yucatán Península, Campeche and Quintana Roo. Two samples were from patients from Jalisco and four samples were sent to the laboratory with limited geographical information (two from Yucatán and two from Quintana Roo). The remaining four samples were obtained from San Juan Laboratories (CDC. San Juan Puerto Rico). These samples were described as being obtained in México, but no specific geographic information was provided.

At the time the first (acute) blood samples were taken, a clinical/epidemiological questionnaire was filled out for each one of the patients by a physician in the Centro de Investigaciones Regionales, who also conducted a physical examination. The clinical information obtained from the patients was stored in a database that is kept at the Universidad Autónoma de Yucatán.

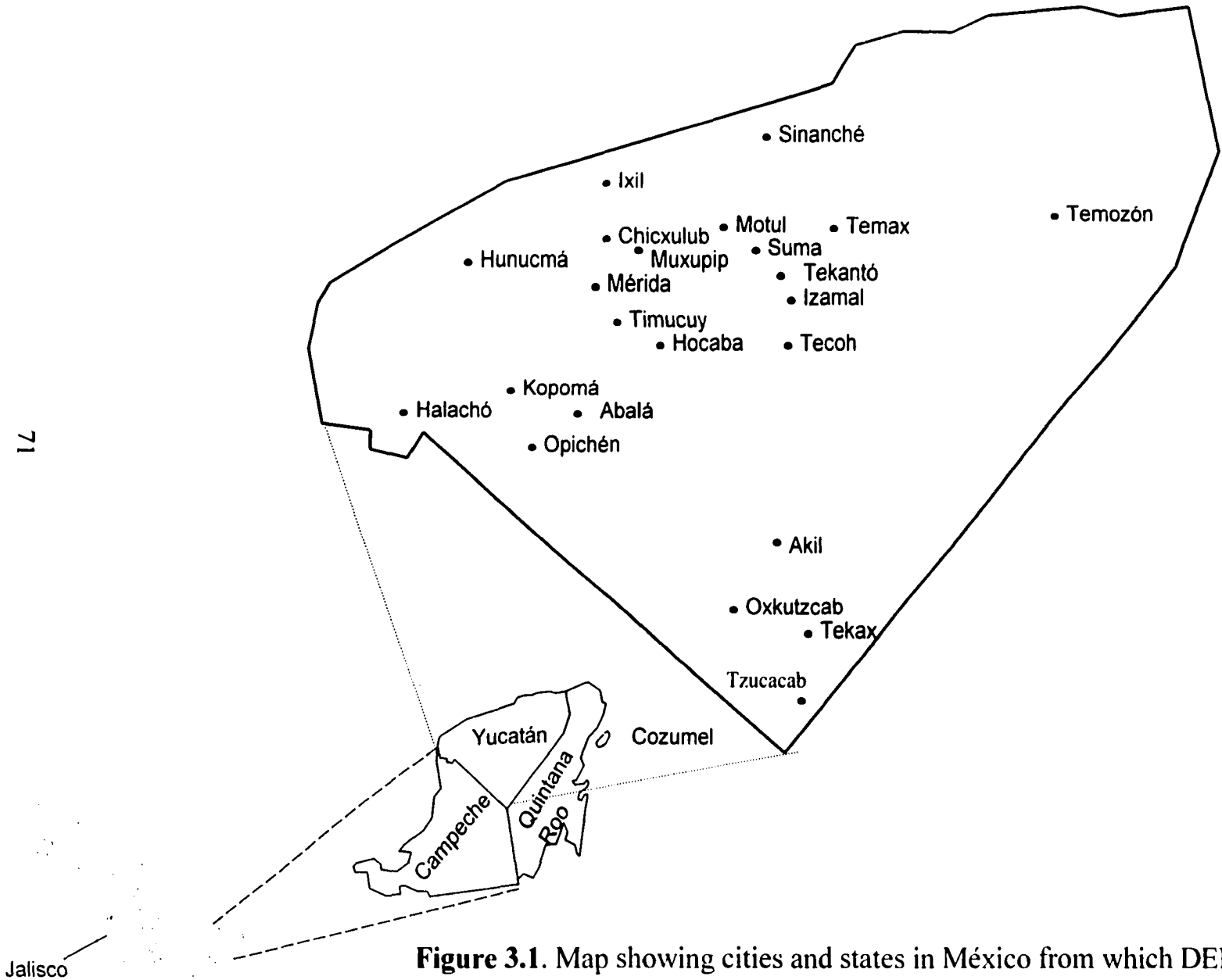


Figure 3.1. Map showing cities and states in México from which DEN viruses originated for this study. Each location is a site where virus was transmitted to patients.

SSCP analysis

Viruses were characterized by SSCP analysis using the procedure described in Chapter 2. SSCP analysis of the prM gene was conducted with viruses from each of the four serotypes. The collection of viruses analyzed was comprised of 23 dengue-1, 10 dengue-2, 62 dengue-3 and 45 dengue-4 isolates (Table 3.1). The information about site of residence of the patients, year that the viruses were transmitted, clinical diagnosis and haplotype of the viruses by SSCP, is listed on the Tables 3.2 to 3.5. SSCP haplotypes were assigned for each of the different banding patterns that are differentiated by SSCP analysis.

Isolates	1980	1983	1984	1992	1994	1995	1996	1997	Total
Dengue-1	1				19	3			23
Dengue-2		1	3	2	3		1		10
Dengue-3						1	30	31	62
Dengue-4			28		1	5	10	1	45
	1	1	31	2	23	9	41	32	140

Table 3.1. Dengue isolates from the Yucatán Península used for the SSCP analysis, organized by the year of isolation.

ISOLATE ID	CITY	STATE	YEAR OF TRANSMIS.	CLINICAL DIAGNOSIS	HAPLOTYPE
BC159/95	MERIDA	YUCATAN	1980	DF	I
BC136/94	MERIDA	YUCATAN	1994	DFHM	II
BC137/94	MERIDA	YUCATAN	1994	DFHM	II
BC138/94	MERIDA	YUCATAN	1994	DFHM	III
BC120/94	MERIDA	YUCATAN	1994	DF	III
BC131/94	MERIDA	YUCATAN	1994	DF	IV
BC132/94	MERIDA	YUCATAN	1994	DF	III
BC119/94	MERIDA	YUCATAN	1994	DF	V
BC121/94	MERIDA	YUCATAN	1994	DFHM	II
BC125/94	MERIDA	YUCATAN	1994	DF	III
BC126/94	MERIDA	YUCATAN	1994	DF	III
BC127/94	MERIDA	YUCATAN	1994	DFHM	III
BC128/94	MERIDA	YUCATAN	1994	DFHM	VI
BC129/94	MERIDA	YUCATAN	1994	DF	III
BC140/94	MERIDA	YUCATAN	1994	DF	III
BC141/94	MERIDA	YUCATAN	1994	DF	III
BC260/95	MERIDA	YUCATAN	1994	DF	III
BC261/95	MERIDA	YUCATAN	1994	DF	III
BC262/95	MERIDA	YUCATAN	1994	DF	III
BC263/95	MERIDA	YUCATAN	1994	DF	III
BC3/96	CAMPECHE	CAMPECHE	1995	DF	V
BC7/96	COZUMEL	QUINTANA ROO	1995	DFHM	III
BC264/95	CAMPECHE	CAMPECHE	1995	DF	III

Table 3.2. Place of origin, year of transmission, diagnosis and haplotype of 23 dengue-1 isolates used for the SSCP analysis. DF= Dengue Fever, DHF= Dengue Hemorrhagic Fever, DFHM= Dengue Fever with Hemorrhagic Manifestations, U=Unknown.

ISOLATE ID	CITY	STATE	YEAR OF TRANSMIS.	CLINICAL DIAGNOSIS	HAPLOTYPE
BC133/94	.	QUINTANA ROO	1994	DF	I
BC139/94	.	QUINTANA ROO	1994	DFHM	I
BC134/94	MERIDA	YUCATAN	1994	DFHM	II
BC17/97	MERIDA	YUCATAN	1996	DF	III
BC122/94	.	JALISCO	1992	DF	IV
BC123/94	.	JALISCO	1992	DF	IV
BC165/95	.	.	1983	U	I
BC200/95	.	.	1984	U	I
BC191/95	.	.	1984	U	I
BC199/95	.	.	1984	U	I

Table 3.3.Place of origin, year of transmission, diagnosis and haplotype of 10 dengue-2 isolates used for the SSCP analysis.

ISOLATE ID	CITY	STATE	YEAR OF TRANSMIS.	CLINICAL DIAGNOSIS	HAPLOTYPE
BC4/96	MERIDA	YUCATAN	1995	DF	I
BC19/97	TEMAX	YUCATAN	1996	DF	I
BC20/97	TEMAX	YUCATAN	1996	DF	I
BC21/97	MERIDA	YUCATAN	1996	DF	I
BC22/97	MERIDA	YUCATAN	1996	DF	I
BC23/97	MERIDA	YUCATAN	1996	DF	I
BC24/97	MERIDA	YUCATAN	1996	DFHM	I
BC25/97	MOTUL	YUCATAN	1996	DF	I
BC27/97	TEMAX	YUCATAN	1996	DF	I
BC28/97	MERIDA	YUCATAN	1996	DFHM	I
BC29/97	MERIDA	YUCATAN	1996	DFHM	I
BC30/97	HUNUCMA	YUCATAN	1996	DF	I
BC35/97	MERIDA	YUCATAN	1996	DF	I
BC36/97	HUNUCMA	YUCATAN	1996	DF	I
BC37/97	OXKUTZKAB	YUCATAN	1996	DF	I
BC38/97	IZAMAL	YUCATAN	1996	DF	I
BC179/97	MOTUL	YUCATAN	1996	DF	I
BC180/97	SINANCHE	YUCATAN	1996	DF	I
BC181/97	MOTUL	YUCATAN	1996	DF	I
BC182/97	AKIL	YUCATAN	1996	DF	I
BC183/97	AKIL	YUCATAN	1996	DF	I
BC184/97	AKIL	YUCATAN	1996	DF	I
BC203/97	MOTUL	YUCATAN	1996	DF	I
BC200/97	MOTUL	YUCATAN	1996	DF	I
BC205/97	MERIDA	YUCATAN	1996	DF	I
BC206/97	TEKAX	YUCATAN	1996	DF	I
BC208/97	IXIL	YUCATAN	1996	U	I
BC283/97	MUXUPIP	YUCATAN	1996	DF	I
BC284/97	IZAMAL	YUCATAN	1996	DF	I
BC285/97	HUNUCMA	YUCATAN	1996	DF	II
BC286/97	CHICXULUB	YUCATAN	1996	DF	I
BC156/97	HOCABA	YUCATAN	1997	DF	I
BC157/97	.	YUCATAN	1997	DF	I
BC158/97	MERIDA	YUCATAN	1997	DF	I
BC159/97	MERIDA	YUCATAN	1997	DFHM	I
BC178/97	TIMUCUY	YUCATAN	1997	DHF	I
BC186/97	MERIDA	YUCATAN	1997	DF	I
BC187/97	MERIDA	YUCATAN	1997	DF	I
BC188/97	MERIDA	YUCATAN	1997	DF	I
BC189/97	MERIDA	YUCATAN	1997	DFHM	I
BC190/97	SUMA	YUCATAN	1997	DF	I
BC191/97	.	YUCATAN	1997	U	I
BC192/97	HOCABA	YUCATAN	1997	DF	III
BC194/97	HOCABA	YUCATAN	1997	DF	I
BC195/97	TECOH	YUCATAN	1997	DF	IV
BC196/97	HOCABA	YUCATAN	1997	DF	I
BC199/97	HOCABA	YUCATAN	1997	DF	I
BC288/97	OXKUTZCAB	YUCATAN	1997	DF	I
BC289/97	MERIDA	YUCATAN	1997	DF	I
BC290/97	OPICHEN	YUCATAN	1997	DFHM	I
BC291/97	HOCABA	YUCATAN	1997	DF	I

Table 3.4. Place of origin, year of transmission, diagnosis and haplotype of 62 dengue-3 isolates used for the SSCP analysis.

ISOLATE ID	CITY	STATE	YEAR OF TRANSMIS.	CLINICAL DIAGNOSIS	HAPLOTYPE
BC292/97	HOCABA	YUCATAN	1997	DF	I
BC294/97	MERIDA	YUCATAN	1997	DF	I
BC295/97	TEMOZON	YUCATAN	1997	U	I
BC296/97	HOCABA	YUCATAN	1997	DF	I
BC297/97	HOCABA	YUCATAN	1997	DF	I
BC298/97	MERIDA	YUCATAN	1997	DF	I
BC299/97	MERIDA	YUCATAN	1997	DF	I
BC300/97	KOPOMA	YUCATAN	1997	DFHM	I
BC301/97	HALACHO	YUCATAN	1997	DFHM	I
BC177/97	CAN CUN	QUINTANA ROO	1997	DFHM	I
BC197/97	.	QUINTANA ROO	1997	DF	I

Table 3.4. (Continued).

ISOLATION ID	CITY	STATE	YEAR OF TRANSMIS.	CLINICAL DIAGNOSIS	HAPLOTYPE
BC146/95	MERIDA	YUCATAN	1984	DF	I
BC147/95	MERIDA	YUCATAN	1984	DF	IV
BC148/95	MERIDA	YUCATAN	1984	DF	I
BC149/95	MERIDA	YUCATAN	1984	DF	I
BC155/95	MERIDA	YUCATAN	1984	DF	I
BC156/95	MERIDA	YUCATAN	1984	DF	I
BC157/95	MERIDA	YUCATAN	1984	DF	I
BC158/95	MERIDA	YUCATAN	1984	DF	I
BC142/95	MERIDA	YUCATAN	1984	DFHM	II
BC143/95	MERIDA	YUCATAN	1984	DF	II
BC144/95	MERIDA	YUCATAN	1984	U	II
BC145/95	MERIDA	YUCATAN	1984	DF	II
BC150/95	MERIDA	YUCATAN	1984	U	II
BC180/95	MERIDA	YUCATAN	1984	U	II
BC187/95	MERIDA	YUCATAN	1984	U	II
BC195/95	MERIDA	YUCATAN	1984	DF	IV
BC196/95	MERIDA	YUCATAN	1984	DFHM	IV
BC197/95	MERIDA	YUCATAN	1984	DFHM	II
BC201/95	MERIDA	YUCATAN	1984	DF	II
BC202/95	MERIDA	YUCATAN	1984	DFHM	II
BC203/95	MERIDA	YUCATAN	1984	DF	II
BC204/95	MERIDA	YUCATAN	1984	U	II
BC205/95	MERIDA	YUCATAN	1984	DF	II
BC206/95	MERIDA	YUCATAN	1984	DF	II
BC207/95	MERIDA	YUCATAN	1984	DF	III
BC208/95	MERIDA	YUCATAN	1984	DFHM	II
BC209/95	MERIDA	YUCATAN	1984	DF	II
BC210/95	MERIDA	YUCATAN	1984	DF	II
BC135/94	MERIDA	YUCATAN	1994	DF	I
BC2/96	OXCUTZCAB	YUCATAN	1995	DF	V
BC5/96	OXCUTZCAB	YUCATAN	1995	DF	V
BC6/96	ABALA	YUCATAN	1995	DF	VIII
BC8/96	B.JUAREZ	QUINTANA ROO	1995	DF	VI
BC9/96	MERIDA	YUCATAN	1995	DFHM	I
BC10/96	OXKUTZCAB	YUCATAN	1995	DF	VII
BC11/96	.	QUINTANA ROO	1995	DF	V
BC24/96	OXKUTZCAB	YUCATAN	1995	DF	V
BC18/97	MERIDA	YUCATAN	1996	DF	VI
BC26/97	IZAMAL	YUCATAN	1996	DFHM	VI
BC185/97	TEKANTO	YUCATAN	1996	DF	VI
BC201/97	CHICXULUB	YUCATAN	1996		I
BC204/97	CHICXULUB	YUCATAN	1996		I
BC207/97	CHICXULUB	YUCATAN	1996		I
BC293/97	CHICXULUB	YUCATAN	1996		I
BC287/97	TZUCACAB	YUCATAN	1997		VI

Table 3.5. Place of origin, year of transmission, diagnosis and haplotype of 45 dengue-4 isolates used for the SSCP analysis.

Virus isolation

Virus isolation was performed in C6/36 (*Aedes albopictus)* mosquito cell culture. Briefly, 50 μl of serum was inoculated into a 25 cm^2 Falcon flask containing a confluent monolayer of C6/36 cells grown on Leibovitz (L-15) medium containing 10% fetal calf serum and 100 units penicillin/100 μg streptomycin/ml. Prior to addition of serum, the old medium was removed and replaced with 500 μl of L-15 medium containing 2% fetal calf serum and 100 units penicillin/100 μg streptomycin/ml (maintenance medium). The inoculated flasks were kept for half an hour at room temperature and then 5 ml of maintenance medium was added. The cultures were incubated for 7-10 days at 28 °C. The serotype of each virus was determined by immunofluorescence analysis using serotype specific antibodies (Henchal *et al.*, 1982, 1983).

RNA extraction

Total RNA was extracted from the infected cells with the QIAamp viral RNA kit (QIAGEN). Briefly, 280μl of cell culture suspension was lysed with 1,120 μl of the buffer AVL at room temperature for 10 min. The suspension was mixed with 1,120 μl of ethanol (96-100%), placed in a QIAamp spin column and centrifuged at 6,000 x g for 1 min. The column was washed twice with 500 μl of buffer AW, and centrifuged at 6,000 x g for 1 min, then centrifuged at 20,000 x g for 3 min. The RNA was eluted from the column with 50μl of preheated RNase-free water (80°C) by centrifugation at 6,000 x g for 1 min.

Oligonucleotide primers

The primers used in this study were designed using the Oligo 4.0 software package

(National Biosciences, Inc., Plymouth, MN). In all cases they were selected to amplify cDNAs about 300 nt long and were synthesized by the personnel of Macro Molecular Resources at Colorado State University. In order to select primers for each one of the dengue virus serotypes, the sequences of the following viral strains were used: dengue-1 Singapore S27/90 (Fu *et al.*, 1992), dengue-2 New Guinea C (Irie *et al.*, 1989), dengue-3 H-87 (Osatomi and Sumiyoshi, 1990) and dengue-4 Dominica (Zhao *et al.*, 1986) The nucleotide sequences of the primers are listed in the Table 3.6.

Primer ID	Sequence	Prod. Length (nt)	Ann. Temp (°C)
dn1prm1**f**	5'-CGTTCCATTTGACTACACGAG-3'	311	54.7
dn1prm1**r**	5'-TAGACCAAGTCCCACGTGTGG-3'		
dn2prm1**f**	5'-ACCACACGTAACGGAGAACCA-3'	291	54.7
Dn2prm1**r**	5'-TCCCACATGTGGAACGAGTGC-3'		
dn3prm1**f**	5'-GCAACACTTGCTTTCCACTTA-3'	287	53.4
dn3prm1**r**	5'-GATCTCTTATCGCGTCTATGC-3		
dn4prm1**f**	5'-GTCAACAAGAGATGGCGAACC-3'	284	53.4
dn4prm1**r**	5'-GTGGTGTTAAAGCTACTGAGC-3'		

Table 3.6. Primers used for the SSCP analysis of the dengue virus isolates, length of the cDNAs amplified with each pair and their respective annealing temperature.
f= forward primer, **r**=reverse primer

Forward primers for prM region of dengue virus 1, 2, 3 and 4 were identical to nucleotides 421 to 441, 372 to 392, 425 to 445 and 449 to 469, respectively, of the corresponding sequence. Reverse primers were complementary to nucleotides 711 to 731, 642 to 662, 691 to 711 and 712 to 732, respectively.

Synthesis of cDNA

cDNA was reverse transcribed using the SuperScript II RNase H Reverse Transcriptase kit (Life Technologies, Gaithersburg, MD). Briefly, for each reaction, RNA and 50 to 100 picomoles of the dengue serotype-specific reverse primers were denatured at 70°C for 10 min and placed on ice before addition of 500 µM each deoxynucleotide triphosphates (dNTP) and 200 units of SuperScript II in 50 mM Tris-HCl (pH 8.3 at 25°C), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol (DTT). The final reaction volume was 20 µl. The samples were incubated for 50 min at 42°C before heating the reaction for 15 min at 70°C to inactivate the enzyme. The sequence of the reverse primers specific for each one of the dengue serotypes is listed in Table 3.6.

PCR

Two µl of first-strand cDNA reaction was amplified in a total volume of 50 µl containing 50 picomoles of each forward and reverse primers in 10 mM Tris-HCl (pH 8.3), 50mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 µM of each dNTP and 2.5 U of the Taq polymerase (Promega, Madison, WI). The PCR reaction was performed in a Touchdown thermocycler (Hybaid Limited, Middlesex, UK) that incubated samples at 94°C for 1 min, 2 min at the calculated annealing temperature for each one of the primer pairs (Table 3.6)

for primer annealing, and at 72°C for 3 min for primer extension, for 30 cycles, followed by incubation at 72°C for 7 min. The amplified products were visualized by electrophoresis on 2% agarose gels with ethidium bromide, using TAE (40 mM Tris-acetate, 1 mM EDTA) as the electrophoresis buffer. The bands were visualized under UV light.

Single-strand conformation polymorphism analysis

SSCP analysis was performed using the procedure described in Chapter 2 (Methods & Materials).

Statistical analysis

The diversity of haplotypes was analyzed using the Keefe and Bergersen's diversity index T (Keefe, 1977), where: $T= 1- \Sigma (p_i)^2$, and p_i is the proportion of the haplotype i among all haplotypes in the group. The diversity index was compared among serotypes. For this analysis, the unbiased estimator $TU=1(n/(n-1))\{\Sigma(p_i)^2-(1/n)\}$ was used. Viruses from the Yucatán Peninsula, 2 viruses from the State of Jalisco and 4 viruses from unknown places in México (Tables 3.2 to 3.5) were analyzed. This last 4 samples were obtained from the CDC, Division of Vector Borne Infectious Diseases, San Juan Laboratories, Dengue Branch in San Juan, Puerto Rico. The genetic diversity (number of haplotypes) of each dengue virus serotype was determined and compared. To assess the significance of the difference between two diversity indexes, the Z test was used. $Z=(TU1-TU2)/\{(\sigma_1)^2/n_1+(\sigma_2)^2/n_2\}^{1/2}$. Calculations were conducted using the program DENGDIVR provided by Dr. William C. Black IV.

RESULTS

Analysis of SSCP haplotypes diversity in geographic areas

cDNAs from the prM locus were synthesized and amplified by RT-PCR with each primer set. The size of each cDNA obtained from the dengue viruses from México were analyzed by agarose gel electrophoresis prior to SSCP analysis. All of the cDNAs were of the size predicted for RT/PCR amplification.

Six distinct SSCP patterns were identified from the 23 dengue-1 isolates obtained from states comprising the Yucatán Peninsula. The dengue-1 virus isolated in 1980 from Mérida, Yucatán formed 1 of the 6 SSCP haplotypes. Fifteen of the 23 dengue-1 viruses displayed identical SSCP patterns (dengue-1 SSCP haplotype 3; Figure 3.2). Thirteen of the 15 dengue-1 SSCP haplotype 3 viruses were isolated in 1994 from patients in Mérida, Yucatán and the other two in 1995 from patients in the adjacent states of Campeche and Quintana Roo. Two of the 23 dengue-1 viruses generated identical, but distinct SSCP patterns, and were designated dengue-1 SSCP haplotype 5 (Figure 3.2). The two dengue-1 SSCP haplotype 5 isolates were transmitted in 1994 and 1995 to patients from Mérida, and Campeche state, respectively. Four other dengue-1 viruses from the 1994 epidemic in Mérida gave unique SSCP patterns. Although the number of dengue-2 virus isolates in the collection was limited, we were able to obtain additional isolates from México through the Vector Borne Division of CDC (Puerto Rico). A total of 10 dengue-2 virus isolates were analyzed by SSCP analysis. Four distinct haplotypes were observed. Interestingly, two dengue-2 isolates transmitted in 1994 in Quintana Roo gave identical SSCP patterns to 4 dengue-2 viruses from México isolated during 1983 and 1984 (no information about

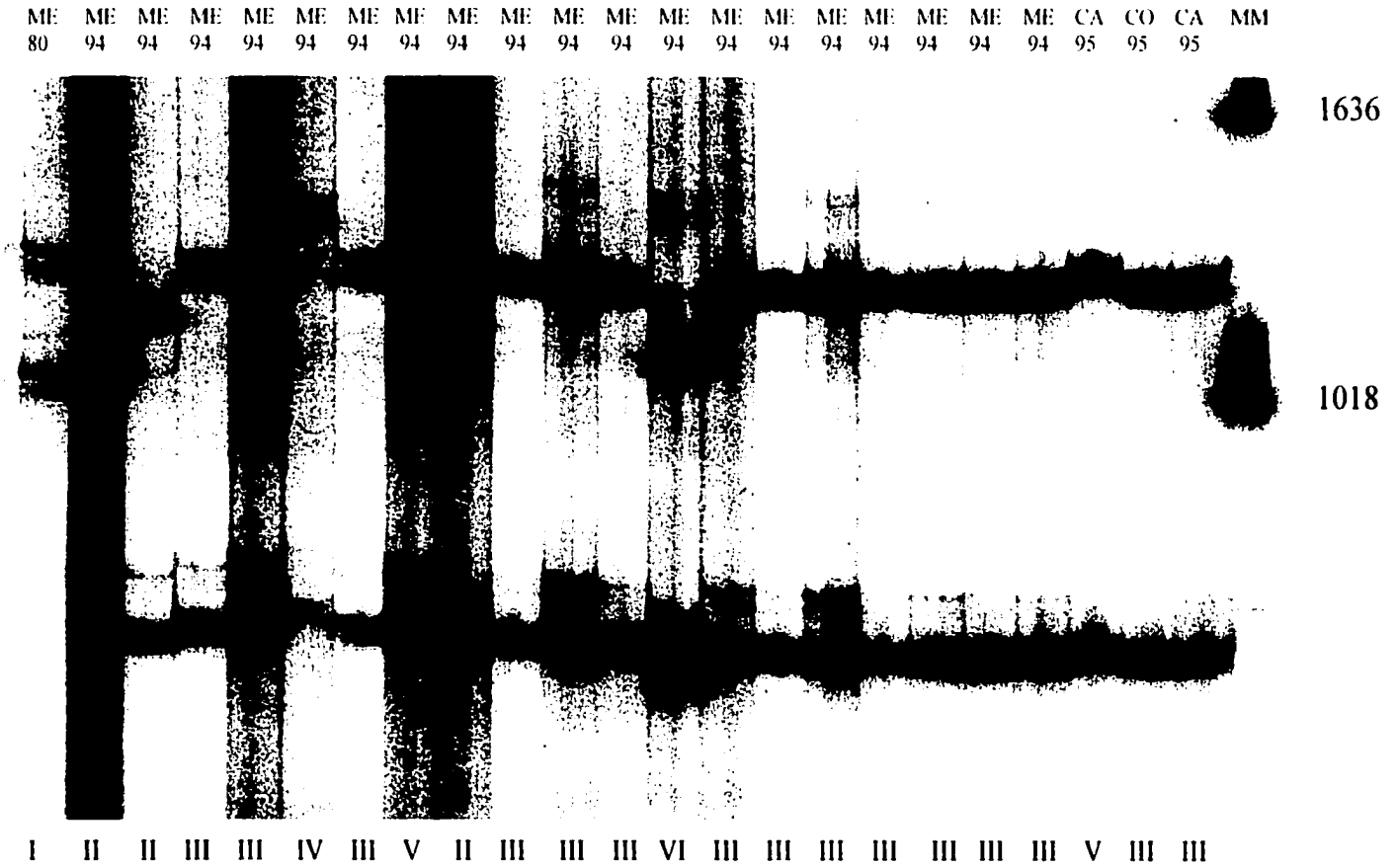


Figure 3.2. SSCP analysis of dengue-1 virus strains from the Yucatán Península. ME = Merida, CA = Campeche, Campeche, CO = Cozumel, Quintana Roo. MM = Molecular marker. See Figure 3.1 for location of transmission sites.The year of transmission is given below the site of transmission. The numbers at the bottom of the SSCP gel indicate the assigned haplotype based on the SSCP pattern.

The virus collection used in this study contained 62 dengue-3 viruses that were isolated from patients who were infected between 1995 and 1997. Sixty of the viruses originated from patients in the state of Yucatán and 2 from patients in Quintana Roo. Only 4 distinct haplotypes among the 62 dengue-3 viruses were observed at the prM locus. Fifty-nine of the viruses displayed identical SSCP patterns and were designated dengue-3 SSCP haplotype 1; 57 of the 59 isolates came from patients in the state of Yucatán. SSCP analysis showed that dengue-3 viruses displaying dengue-3 SSCP haplotype 1 originated throughout Yucatán in 1995 to 1997. The dengue-3 SSCP haplotype 1 viruses originated in Mérida (18 viruses), and cities predominantly east [Hocaba, (8 viruses); Motul (4 viruses) and Temax (3 viruses)] of Mérida (Figure 3.1). Three individual isolates displayed distinct SSCP haplotypes at the prM locus and were designed haplotypes 2, 3 and 4 respectively. Interestingly each one of the three isolates originated from patients in different locations (haplotype 2 in Hunucma, 3 in Hocaba and 4 in Tecoh 4). Two dengue-3 SSCP haplotype 1 viruses were transmitted in Izamal (east of Merida), Oxkutzcab and Akil (south of Merida) and Hunucma (East of Merida).

The virus collection also contained 28 dengue-4 viruses isolated from patients in Mérida in 1984. This was the first year this dengue virus serotype was identified in the state of Yucatán. Seventeen of the 28 dengue-4 viruses displayed identical SSCP patterns at the prM locus and were designated dengue-4 haplotype 2. Seven dengue-4 1984 isolates were designated dengue-4 SSCP haplotype 1. Three dengue-4 isolates were designated dengue-4 SSCP haplotype 4, and another isolate was designated dengue-4 SSCP haplotype 3. The collection also contained 17 dengue-4 viruses originating from human

infections that occurred between 1994 and 1997. Dengue-4 SSCP haplotype 1 observed in 1984 was also present after 1994. Five different haplotypes were observed from this group of dengue-4 viruses. Four viruses from patients in Chicxulub and two Merida were of dengue-4 SSCP haplotype 1. dengue-4 viruses from B. Juarez, Mérida, Izamal, Tekantó and Tzucacab, gave identical SSCP patterns and were designated dengue-4 SSCP haplotype 4. Three viruses from Oxkutzcab and one from the Quintana Roo were dengue-4 SSCP haplotype 5. Finally, single dengue-4 isolates from Oxkutzcab, and Abala were designated dengue-4 SSCP haplotypes 7, and 8, respectively.

Haplotypes	1980	1994	1995	TOTAL
1	1			1
2		3		3
3		13	2	15
4		1		1
5		1	1	2
6		1		1
TOTAL	1	19	3	23

Table 3.7. Dengue-1 haplotype prevalences in virus isolates by year of transmission in the Yucatán Península.

Haplotypes	1994	1996	TOTAL
1	2		2
2	1		1
3	1	1	1
TOTAL	3	1	4

Table 3.8. Dengue-2 haplotype prevalences in virus isolates by year of transmission in the Yucatán Península

Haplotypes	1995	1996	1997	TOTAL
1	1	29	22	59
2		1		1
3			1	1
4			1	1
TOTAL	1	30	31	62

Table 3.9. Dengue-3 haplotype prevalences in virus isolates by year of transmission in the Yucatán Península

Haplotypes	1984	1994	1995	1996	1997	TOTAL
1	7	1	1	4		13
2	17					17
3	1					1
4	3					3
5			4			4
6			1	3	1	5
7			1			1
8			1			1
TOTAL	28	1	8	7	1	45

Table 3.10. Dengue-4 haplotype prevalences in virus isolates by year of transmission in the Yucatán Península.

Statistical analysis of SSCP haplotype diversity

To analyze diversity of dengue SSCP haplotypes, the Keefe and Bergersen diversity index for haplotypes within each serotype were calculated (Table 3.11). The diversity indexes between each pair of a serotype (1 vs. 3, 1 vs. 4 and 3 vs. 4) was compared. For this comparison, dengue-2 haplotypes were excluded due to the small number of isolates (dengue-2, 10 isolates).

The diversity of SSCP haplotypes in dengue-2 was the highest (0.6444) followed

by dengue-4 (0.5771), dengue-1 (0.5691) and dengue-3 (0.0951). Comparison of diversity indices of SSCP haplotypes for dengue-1, dengue-3 and dengue-4 revealed that dengue-3 diversity index was dramatically lower and differed significantly from those for dengue-1 and dengue-4.

Keefe-Bergeson analysis of differences among dengue virus serotypes

Serotype	n		TU	Number of haplotypes
1	23		0.5691	6
2	10		0.6444	4
3	62		0.0951	4
4	45		0.5771	8

Serotype		Serotype	Z
1	vs.	3	3.8338 *
1	vs.	4	0.0601
3	vs.	4	5.5278 *

Table 3.11. Keefe Bergeson diversity index (TU) among haplotypes in the 4 dengue virus serotypes. * $p \leq 0.05$.

DISCUSSION

Among Flaviviruses, the prM gene is intermediate in variability when compared with other genes of the viruses (Blok *et al.*, 1992). The prM sequence is about twice as variable as NS5, the putative polymerase gene of dengue viruses, but less variable than NS2a, a more variable gene among the Flaviviruses (Blok *et al.*, 1992; Westaway and Blok, 1997). SSCP analysis of the prM fragment yielded more discriminating haplotypes among the different virus isolates than the fragments of the E and NS5 coding genes(Chapter 2) (Farfan *et al.*, 1997). Apparently, the intermediate level of genomic variability of the prM gene made this region a good marker to assess genetic variability among dengue virus

isolates in México. Interestingly, specific changes in the nucleotide sequence of the prM gene have been associated with enhanced virulence of dengue viruses (Thant *et al.*, 1996; Rico-Hesse *et al.*, 1999).

It has been reported that the sensitivity of SSCP can reach the point of detecting a single nucleotide replacement. In these studies this level of detection can be achieved with some sequences, but not with all. However, considering the rapidity of the technique and the low cost of the of the procedure (compared to genome nucleotide sequencing), the RT-PCR SSCP is a good screening procedure to identify the introduction of new variants of dengue viruses in endemic areas. In this study, we have used SSCP to assess genetic variation within the prM coding region of dengue viruses (serotypes 1-4) in México. The viruses used in this analysis were obtained from a virus collection that originated from human patients in Yucatán, México and surrounding states between 1980 and 1997.

It is noteworthy that analysis of the dengue-1 and dengue-3 isolates revealed that the SSCP haplotype most abundant in one year was the most likely haplotype to present the following year. For dengue-1, haplotype 3 comprised 13 of the 19 dengue-1 virus isolates transmitted in 1994 (68%) and 2 out of 3 (66%)the following year. Similarly for dengue-3, haplotype 1 was transmitted most abundantly during 1996 (30 of 31 isolates). In 1997, the same haplotype was again the more prevalent (29 of 31 isolates).

The virus collection also contained 28 dengue-4 viruses isolated from patients in Mérida in 1984. This was the first year this dengue virus serotype was identified in the state of Yucatán. Seventeen of the 28 dengue-4 viruses displayed identical SSCP patterns at the prM locus and were designated dengue-4 haplotype 2. Seven of the 1984 dengue-4

isolates were designated SSCP haplotype 1. Three dengue-4 isolates were designated haplotype 4, and another isolate was designated haplotype 3. The collection also contained 17 dengue-4 viruses originating from human infections that occurred between 1994 and 1997. Dengue-4 SSCP haplotype I (observed in 1984) was also present after 1993. Five different haplotypes were observed from this group of dengue-4 viruses. Four dengue-4 viruses from patients in Chicxulub and two from Mérida were haplotype 1. Certain dengue-4 viruses from B. Juárez, Mérida, Izamal, Tekantó and Tzucacab gave identical SSCP patterns and were designated haplotype 4. Three viruses from Oxkutzcab and one from the Quintana Roo were designated haplotype 4. Finally, single dengue-4 isolates from Oxkutzcab and Abalá were designated dengue-4 SSCP haplotypes 7 and 8, respectively.

SSCP is an easy, rapid, and sensitive means of detecting genetic variation at specific loci within the genomes of many viruses. SSCP has been used to detect genetic variation among hepatitis C, dengue-2, La Crosse, human immunodeficiency virus-1, herpes-1 and 2, and papilloma viruses (Xi *et al.*, 1993; Black *et al.*, 1995; Lin *et al.*, 1995; Baron *et al.*, 1996; Farfan *et al.*, 1997; Chandler *et al.*, 1998). Previously, we used SSCP to analyze two 291 nucleotide loci within the prM and E coding regions of 19 dengue-2 viruses isolated between 1972 and 1974 from patients inhabiting three islands in the Pacific (Farfan *et al.*, 1997). When the E locus was analyzed, all the banding patterns were identical regardless of the place or year of origin of the dengue-2 virus strain. This showed that the E locus chosen in this study had low genetic variation and clearly demonstrated the reproducibility of SSCP analyses. In contrast, the same 19 dengue-2 isolates displayed

3 distinct SSCP patterns at the prM locus. Three different SSCP patterns were displayed among the 4 viruses from Samoa. The 10 viruses from Tonga and 5 viruses from Tahiti had identical SSCP patterns. In addition, SSCP analyses of 12 dengue-2 virus isolates from representative topotype groups revealed that the prM locus was more variant than the E locus (Farfan *et al.*, 1997).

Our studies suggest that RT-PCR/SSCP is an excellent method of observing the introduction of new dengue viruses into endemic areas of México. For instance, SSCP analysis clearly showed that a predominant SSCP haplotype (haplotype 1) of dengue-3 virus was present in Yucatán in 1995-1997 and may reflect the dengue-3 haplotype originally introduced in that geographic region. In addition, SSCP analysis of dengue-4 viruses also suggests a prominent haplotype (haplotype 2) was circulating in Mérida, Yucatán in 1984.

Most of the isolates analyzed were from the Yucatán Península, and the low diversity index corresponding to dengue-3 haplotypes could be the result of the history of this serotype in the Península. Dengue-3 was identified for the first time in Yucatán in September of 1995. In contrast, dengue-1 and dengue-4 were identified in 1979 and 1984, respectively. The reduced number of dengue-3 haplotypes may be the result of a reduced number of parent haplotypes that were introduced into the Península and that are still at an early time in diversification. Dengue-1 and dengue-4 could have been introduced many times and/or they may have been in the Península and diversifying for a longer time than dengue-3.

It was not possible to correlate specific haplotypes and dengue disease outcomes

since only one of the isolates originated from a patient displaying all of the symptoms that meet the WHO criteria for diagnosis of DHF. Twenty-five dengue virus isolates originated from patients showing hemorrhagic manifestations; however, none of these fulfilled the criteria leading to diagnosis of DHF.

This study has begun to provide a catalog of the SSCP viral haplotypes that have circulated in the Yucatán Península from the early 1980's to the present. The number of specimens is limited, and in some years, no isolate or only few isolates were obtained. However, this collection of dengue virus strains from México is very important because the isolates were obtained prospectively in a defined geographic area. Typing of the virus isolates by SSCP analysis provides an invaluable resource for continued surveillance of dengue viruses in Yucatán by this technique. This will also permit rapid monitoring for the introduction of novel haplotypes into the Yucatán Península of México.

CHAPTER 4

EFFECT OF THE MICROENVIRONMENT ON THE DENGUE-4 PRM GENE

INTRODUCTION

RNA viruses exhibit exceptional genomic plasticity (Domingo and Holland, 1997). Most viruses with RNA genomes have very high mutation rates. Mutation rates at the nucleotide level can vary in different viruses, but the overall nucleotide base misincorporation rate is in the range of 10^{-4} to 10^{-5} (Domingo *et al.*, 1995; Domingo and Holland, 1997). The inability of the viral RNA dependent RNA polymerase to copy accurately the parent viral genome results in the generation of a highly heterogeneous population of closely related genomes called a quasispecies (Eigen and Biebricher, 1988; Eigen *et al.*, 1988). In addition, RNA viruses generally lack or have low efficiency of proof reading-repair activities associated with the RNA replicase (Steinhauer *et al.*, 1992). Thus mutations can accumulate rapidly.

Viruses are intracellular microorganisms, and the intracellular space is the microenvironment in which viruses replicate. Arboviruses replicate in vertebrate and invertebrate host cells.

The microenvironment in which RNA viruses replicate could favor the emergence of new viral variants and could be a determinant of their evolutionary potential. Dengue viruses are routinely isolated from patient's serum by amplifying viruses in cell culture (C6/36 cells), mosquitoes, or newborn mice. Once the viruses are isolated, amplified and identified, the isolates are either stored frozen or serially passaged in cell cultures. Virus replication in animals or cell culture can potentially result in the selection of specific viral sub-populations.

For example, serial passage of dengue viruses has been used to select for viral

variants with reduced virulence. These attenuated viruses can potentially be used for vaccines (Eckels *et al.*, 1984; Halstead *et al.*, 1984; Bhamarapravati *et al.*, 1987; Edelman *et al.*, 1994). A comparison of the nucleotide sequence of dengue-2 virus (Thailand 16681) with virus after 53 serial passages in primary dog kidney (PDK) cells revealed 53 nucleotide substitutions in the genome; 27 of these were non-synonymous, and the highest proportion of amino acid substitutions were in the prM protein (Blok *et al.*, 1992). In another report, (Puri *et al.*, 1997) a non-attenuated, dengue-1 virus mutant (45A25 PDK0) was assayed for genetic stability following passage in PDK cells. The entire genome of 45A25 PDK0 was sequenced before and after 27 serial passages. Twenty-five nucleotide replacements were identified; 11 of these were non-synonymous substitutions. Almost half of the amino acid changes were in the E protein and the rest were distributed in NS1, NS2a, NS3, and NS5. Four of the nucleotide changes were located in the 3' non-coding region (Puri *et al.*, 1997). Atenuation was tested in monkeys and human volunteers. The level of attenuation was directly correlated with the passage number in PDK cells. Interestingly, nucleotide substitutions that were chemically induced in 45AZ5 PDK0 were conserved in the viruses throughout the 27 passages in PDK cells. This observation indicated that the changes responsible for attenuation were those that took place when viruses replicated in the PDK cell microenvironment (Puri *et al.*, 1997).

To determine the influence of *in vivo* and *in vitro* passage on dengue viruses, three isolates of dengue-3 were serially passaged in newborn mouse brain, Vero cells (monkey kidney cells) and C6/36 (*Aedes albopictus* cells). The coding region for the structural proteins were then sequenced. All three isolates that were passaged 10 times in neonate

mouse brain displayed mutations in the E gene. Five out of 6 mutations led to non-conservative changes. One of the isolates had only 1 mutation; the others had 2 and 4 nucleotide replacements, respectively. In the passaged virus containing 4 mutations, one was synonymous. The viruses obtained after the tenth passage had increased neurovirulence in mice. Six independent serial virus passages were done in Vero cells. In 4 of the 6 passage series, a single nucleotide replacement occurred at position E-191, in 2 of the 6 passage series, a nucleotide replacement occurred at position E-202, and in 3 of the passage series a replacement occurred at position E-268. Also in 2 of the passage series, changes occurred at position 26 in prM (Lee *et al.*, 1997). In two of the isolates that were serially passaged in C6/36 mosquito cells, there was a change at E-153. It was concluded that passage of dengue-3 virus in mosquito cell culture consistently selects for specific amino acid replacements. However, serial passage in mouse brain selects for diverse amino acid changes. This suggests a greater variety of selective components in the *in vivo* mouse brain system, compared to those in the *in vitro* cell culture microenvironment. Some of the mutations were present in the viruses as early as in the second or fifth passage (Lee *et al.*, 1997).

A 280 nucleotide fragment from the E/NS1 junction of dengue-3 virus from a patient serum was passaged 3X in C6/36 cells and was sequenced. The nucleotide sequences of the virus obtained from the patient serum and from the virus after the serial passage in C6/36 cells were identical (Thayan *et al.*, 1997). Similarly, a 249 nucleotide fragment corresponding to the E/NS1 gene junction of a dengue-2 virus (Puerto Rico strain) was sequenced after 6 passages in primary green monkey kidney cells, after 13 more passages

in the same cell line, and after 4 passages in fetal rhesus monkey lung cells. No nucleotide sequence difference was found (Rico-Hesse, 1990). It is important to point out that the same region in the genome had been chosen by other authors for phylogenetic analysis because presumably is not under selective pressure (Rico-Hesse *et al.*, 1997).

It is a common procedure to propagate viruses in cell lines before they are used for nucleotide sequence determination. In the case of dengue viruses, the *Ae. albopictus* C6/36 cell line is commonly used for virus isolation and amplification. From the studies described above it can be seen that different regions of the virus genome can be selected when the viruses are allowed to replicate in different microenvironments.

Dengue viruses normally replicate in two different hosts, humans and mosquitoes. Presumably the genetic plasticity of the viral genome allows the virus to bridge the phylogenetic gap between vertebrate and invertebrate hosts. Thus viral haplotypes that replicate and amplify in humans may differ from those that replicate and amplify in vectors. Viral RNA purified from human serum is derived from viruses that replicated in human cells. Viral replication in C6/36 mosquito cells would probably yield different viral haplotypes, especially from cells cultivated *in vitro*.

The hypothesis for these studies was that passage of virus isolates in mosquito cells would select for genetically distinct virus sub-populations that could be identified by SSCP and nucleotide sequencing. Studies were conducted to determine the extent of genetic heterogeneity in viruses in the serum of a patient and in the resultant virus after passage twice in the *Aedes albopictus* C6/36 mosquito cells. The level of genetic heterogeneity in the two virus populations (serum and mosquito cells) was assessed by

RT-PCR amplification of RNA obtained from human serum or extracted from infected C6/36 cells followed by cloning of the PCR products and SSCP analysis and nucleotide sequencing of the cDNA of the prM genomic region (Figure 4.1). This approach provided information on the composition of viruses in the sample analyzed (serum or cultured cells) and in the virus populations after virus passage in the two microenvironments.

MATERIAL AND METHODS

Human serum

Human serum was obtained from a 30 year old male from Chicxulub, Yucatán, México. Chicxulub is located 24 km north of Mérida, the capital city of the Yucatán State. The patient was displaying symptoms compatible with classical DF. The date of onset of disease was December 14, 1996.

Virus propagation and isolation

Dengue-4 virus (YU6599) was isolated from the patient's serum by inoculating 30 μl of the serum into a 25 cm^2 flask that had a confluent monolayer of C6/36 cells. Cells were propagated in L-15 culture medium (5% fetal bovine serum, penicillin 100 u/ml, streptomycin 0.1mg/ml in 0.9% NaCl). The infected cell culture was incubated at 28°C. At day 7 post infection (p.i.), the infected cells were harvested and pelleted by centrifugation at 1500 rpm for 3 minutes. The cells were resuspended in phosphate buffered saline (PBS), pH 7.2. Twelve-well spot slides were prepared using 15 μl of cell suspension per spot (Henchal *et al.*, 1983). The slides were air dried, fixed in cold acetone (-20°C) for 15 min and stored at -70°C. To generate viruses with two passages in C6/36 cells, virus was

passaged again in C6/36 cells. Twenty five μl of cell suspension was inoculated into a 25 cm^2 flask containing a confluent monolayer of C6/36 cells.

Immunofluorescence Assay

An immunofluorescence (IF) assay using anti-envelope, serotype specific monoclonal antibodies was used to identify the dengue virus serotype. Slides were incubated in a humidified chamber at 37°C for 30 minutes. The monoclonal antibodies were: D2-1F1-3 (anti-dengue-1), 3H5-1-21 (anti-dengue-2), D6-8A1-12 (anti-dengue- 3) and 1H10-6-7 (anti-dengue-4), and 4G2 (group specific for flaviviruses detection) (Henchal *et al.*, 1983). Slides were washed twice in PBS (pH 7.2) and then incubated with fluorescein labeled goat anti-mouse IgG (H+L) at 37°C for 30 minutes. Slides were rinsed twice in PBS and mounted in glycerol-saline-DABCO (1.4 Diazobicyclo (2,2,2) Octane, Sigma # D-2522).

RNA extraction

Viral RNA was extracted from the serum using the QIAamp viral RNA kit (QIAGEN) following the instructions of the manufacturer. This is a modified guanidinium isothiocyanate protocol using silica based spin columns. Briefly, 280μl of the patient's serum was mixed with 1,120 μl of the buffer AVL and incubated at room temperature for 10 min. The suspension was mixed with 1,120 μl of ethanol (96-100%), placed in a QIAamp spin column, and centrifuged at 6,000 g for 1 min. The column was washed twice with 500 μl of buffer AW and centrifuged at 6,000 x g for 1 min, then centrifugated at 20,000 g for 3 min. The RNA was eluted from the column with 50μl of preheated RNase-

free water (80°C), followed by centrifugation at 6,000 x g for 1 min. In the case of infected cell cultures, 280 μl of cell culture suspension was mixed with the AVL lysis buffer instead of serum. The purified RNAs were then quantified spectrophotometrically.

Primer Design

Primers for succeeding reactions were designed using previously determined sequences available in the GenBank DNA sequence database using Oligo™ version 4.0 computer software. General parameters proposed by Sharrocks for primer design were followed (Sharrocks, 1994). Primers DN4PRM1F and DN4PRM1R were used for RT-PCR starting from RNA purified from serum and C6/36 cells and for PCR amplification of the DNA corresponding to the prM gene. Primers D4-357 and D4-822 were used for sequencing of the prM gene region (Table 4.1).

RT-PCR

To amplify cDNA from the viruses in serum and cell culture, the Access RT-PCR System™ (Promega, Madison, WI) was used. This RT-PCR system incorporates avian myeloblastosis virus reverse transcriptase (AMV RT) for first strand cDNA synthesis and *Thermus flavus* Tfl DNA Polymerase for second strand cDNA synthesis and DNA amplification. The system includes an optimized single-buffer system that permits extremely sensitive detection of RNA transcripts without a requirement for buffer additions between the reverse transcriptase and PCR amplification steps. RT-PCR amplification was performed in 50μl reactions containing 10μl AMV/Tfl 5X Reaction Buffer, 0.2mM dNTPs, 1 mM 25mM $MgSO_4$, 5u AMV reverse transcriptase, 5u Tfl DNA polymerase, 50 pmol of

each primer and 1-2 μg of sample RNA. For this procedure, the primers DN4PRM1F and DN4PRM1R were used. The sequences of these primers are listed in Table 4.1.

Table 4.1. Primers used for SSCP analysis and sequencing of the dengue virus prM gene fragments.

Primer	Sequence (5'→3')	Location (5' end)	Product Length	Annealing Temp °C
DN4PRM1f	GTCAACAAGAGATGGCGAACC	449	284	53.4
DN4PRM1r R	GTGGTGTTAAAGCTACTGAGC	732		
D4-357	GAGATAGGCCGCATGCTGAAC	357	486	57.3
D4-822	GAGATAGGCCGCATGCTGAAC	842		

For the RT-PCR, the reaction mixture was placed in a Touchdown thermocycler (Hybaid Limited, Middlesex, UK) programmed to incubate for 45 minutes at 48°C, 2 minutes at 94°C, followed by 40 cycles of denaturation at 94°C for 30 seconds, primer annealing at 53.4°C for 1 minute, and primer extension at 68°C for 2 minutes. The cycling sequence was followed by a final incubation for 7 minutes at 68°C. The cDNAs obtained were 284 nucleotides in length, which is an excellent size for the SSCP procedure.

SSCP analysis

SSCP analysis was performed using the procedure previously described in Chapter 2 (Page 45).

Cloning of RT-PCR products

To assess genetic diversity induced by the passage of dengue-4 viruses that are

propagated in two different microenvironments (human and C6/36 cells), RT-PCR cDNAs generated were initially cloned into a plasmid vector pCR2.1 (Invitrogen, Carlsbad, CA). The DNA from several of these clones was analyzed by SSCP and sequencing. The schematic diagram of the experimental procedure for the RT-PCR, cloning, and SSCP analysis is presented in Figure 4.1.

The cDNA produced in the RT-PCR reaction was cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA). Briefly, ligation was conducted in 10 μl reaction. One μl of cDNA obtained from the RT-PCR reaction was mixed with 50 ng of pCR 2.1 vector and 4 Weiss units of T4 DNA ligase. The mixture was incubated at 14°C for 4 hours. For transformation, 50 μl of One Shot™ *E. coli* cells were mixed 2 μl of ß-mercaptoethanol, followed by the addition of 2 μl of the ligation reaction. The vial containing the mixture was incubated on ice for 30 min. The cells were heat shocked for 30 seconds at 42°C and placed on ice for 2 min. Two hundred and fifty μl of SOC medium (trypticase peptone 2 %, yeast extract 0.5%, NaCl 10mM, KCl 2.5 mM $MgCl_2$-$6H_20$ 10 mM, glucose 20mM) was added, and the vial was shaken at 37°C for one hour. Cells were spread on Luria Bertani (LB) agar plates (Tryptone 1 %, yeast extract 0.5%, 1% NaCl) containing 50 μg/ml of ampicillin and X-Gal (5-Bromuro-4-chloro-3-indolyl-beta-D-galactopyranoside). The plates were incubated at 37°C for at least 14 hours. The presence of the insert was screened by PCR, using the primers DN4PRM1F and DN4PRM1R (Table 4.1). The PCR product of positive clones was analyzed by SSCP.

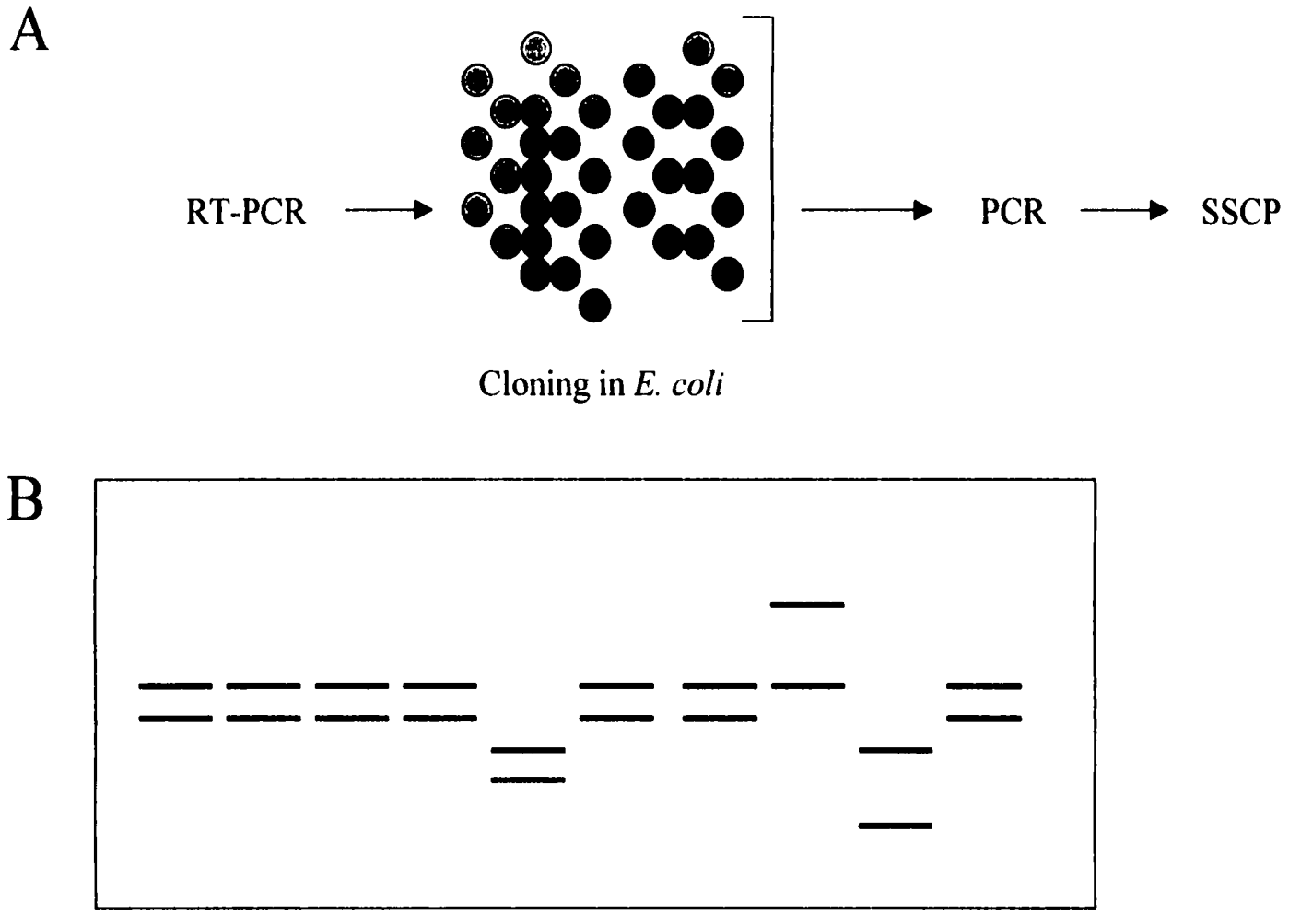


Figure 4.1. A. Protocol used to asses the presence of genomic diversity in the population of viruses present in C6/36 cells and **B**. schematic of a SSCP gel showing the diversity of the banding patterns obtained from different clones.

DNA Sequencing

cDNAs from viruses in serum and C6/36 cells were amplified by RT-PCR using primers that were derived from the genome sequence of dengue-4 strain Dominica (Zhao *et al.*, 1986). The primers (D4-357 and D4-822) were designed to anneal to viral sequences 5' and 3' to the prM gene. D4-357 and D4-822 primer sequences, their annealing temperatures, and the size of the PCR product are listed in Table 4.1. Primers UPF and UPR, which anneal to the pCR2.1 vector, were used to sequence the cDNA. The primers, annealing temperatures and size of the PCR product are listed in Table 4.2. cDNA fragments were purified using QIAquick kit (QIAGEN Inc., Santa Clarita, CA) and sequenced using an ABI 377 DNA Sequencer (Perkin-Elmer Inc., Foster City, CA) with Taq FS polymerase (Perkin-Elmer). Sequencing reactions were performed in both directions. Sequencing was conducted at Macromolecular Resources in Colorado State University.

Primer ID	Sequence (5'→3')	Location (5' end)	Product Length	Annealing Temp °C
UPF	GCTATGACCATGATTACGCCAAGCTCG	79 upstream from insert	182 plus insert length	56
UPF	GACGGCCAGTGAATTGTAATACGACTC	103 downstream from insert		

Table 4.2. Primers used for PCR amplify and sequence the DNA corresponding to the prM gene of dengue-4.

Computer Analysis

DNA sequence analyses were performed using Seqman™ (Dnastar Inc. Madison,

WI). Alignment of sequences from different strains of dengue-4 virus was accomplished using Clustal W version 1.7 (Thompson *et al.*, 1994; Higgins *et al.*, 1996) with a gap open penalty of 15, gap extension penalty of 6.66, and window size of 4.

RESULTS

RT-PCR and cloned cDNA analysis by SSCP

The procedure to clone cDNAs derived from RT-PCR amplification had to be conducted two time, because the number of colonies obtained the first time was low. The second time, 7 and 6 were obtained from cDNAs from C6/36 cell and serum respectively. The SSCP banding patterns of the cDNAs derived by RT-PCR amplification of the prM coding region of dengue-4 virus were identical regardless of whether the cDNA was obtained from serum or C6/36 cells (Figure 4.2, lanes Ser and C6). In contrast, SSCP patterns of cloned cDNAs differed. Seven clones obtained from cDNA amplified from C6/36 infected cells and 6 clones obtained from infected serum were analyzed by SSCP. Three of the 6 clones obtained from viruses in infected serum gave identical banding patterns (left lanes 1,2, and 6) to those amplified directly from viral RNAs in serum and C6/36 cells (central lanes Ser and C6). Each of the three remaining cDNAs yielded a distinct SSCP pattern. Cloned cDNAs from 5 of 7 clones of viruses passaged twice in C6/36 mosquito cells also yielded banding patterns (right lanes 1, 2, 3, 4 and 7) that were identical to those from the uncloned cDNAs that were directly amplified from C6/36 and serum (Figure 4.2, central lanes Ser and C6). The remaining two cDNAs yielded a similar banding pattern.

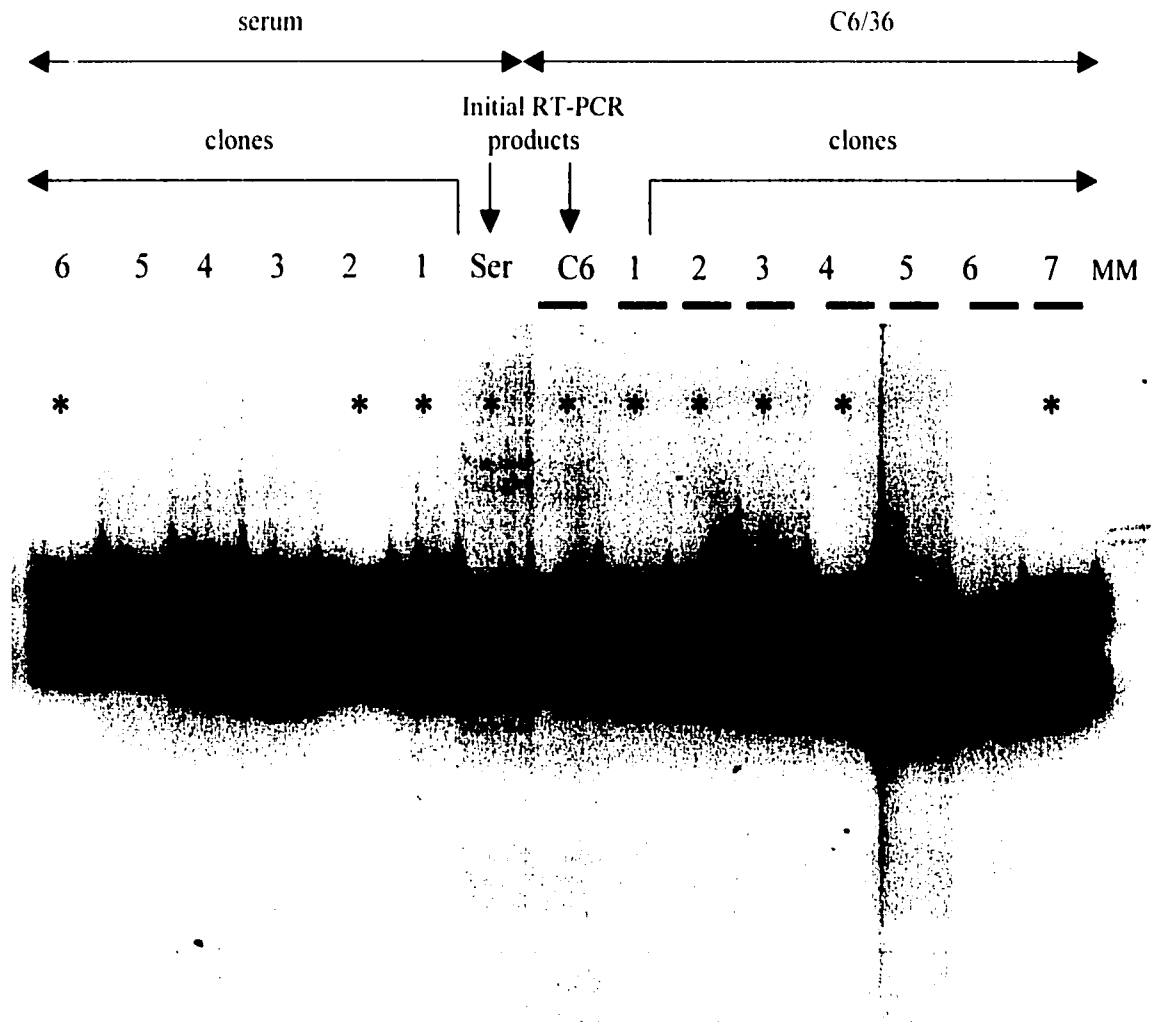


Figure 4.2. SSCP banding patterns of cDNA amplified from dengue virus in serum and after two passages in C6/36 cells.SSCP patterns resulting from cDNA amplified directly from serum (Ser) and C6/36 (C6) were identical. In contrast SSCP bands from 6 individual clones of cDNAs amplified from virus in serum (1-6 left side of the picture) and 7 obtained from virus that was passaged twice in C6/36 cells (1-7 right side of the picture). Lines with * had similar banding patterns.

Nucleotide sequencing

The nucleotide sequences of the cDNAs directly amplified from the patient serum and from the infected C6/36 cells were identical. However 7 out of 13 of the cloned cDNAs products had nucleotide substitutions (Table 4.3, Figure 4.3). To analyze nucleotide replacements at the level of codons, the 284 nucleotide sequence was put in frame with the viral open reading frame. The first and last nucleotides in the 284 nucleotide sequences were not considered for this analysis.

Seven clones were obtained from the RT-PCR product amplified from the viral RNA extracted from viruses that were passaged 2 times in C6/36 cells. Nucleotide substitutions were found in 3 out of 7 cloned cDNAs from viruses passaged in C6/36 cells. All 3 substitutions were in the first position of the codon. Two of them were transitions and 1 was a transversion. Two out of the 3 non-synonymous substitutions in the cDNA from clones from C6/36 cells infected with dengue-4 virus were non-conservative. One threonine→alanine changed from a polar amino acit to non-polar one. The other mutation (lysine→glutamic acid) changed the charge from positive to negative.

Four out of 6 cDNAs from clones amplified from viruses in serum. One of the 4 had two replacements, and the other 3 had one replacement each. Four out of the 5 nucleotide replacements were located in the third position in the codon and were silent mutations. The deduced amino acid sequences of the cDNAs are listed in Table 4.4.

The only non-synonymous change cysteine->arginine, occured in one of the clones obtained from serum.

All three nucleotide substitutions in virus genomes passaged in cell culture were

non-synonymous. In contrast, only one of 5 substitutions in viruses obtained from serum derived clones was non-synonymous. The overall difference in the observed nucleotide substitutions in each one of two microenvironments was then compared. This difference was not statistically significant (p=0.14, Fisher exact test). This result can be explained by the small sample size. All the clones that gave different SSCP patterns also had differences in their nucleotide sequences.

Table 4.3. Number of nucleotide substitutions in the prM sequence of the dengue-4 genomes amplified from serum, from infected C6/36 cells, and from cloned cDNAs. Ser=cDNA obtained from RNA extracted from virus in serum. C62=cDNA obtained from RNA extracted from virus passaged twice in C6/36 cells. S1 to S7=cDNA obtained from cDNAs from viral RNA obtained from serum.C1 to C7=cDNA obtained from cDNAs from viral RNA obtained from infected cells.

	Ser	C62	C1	C2	C3	C4	C5	C6	C7	S1	S2	S3	S4	S5	S6
Ser															
C6	0														
C1	0	0													
C2	0	0	0												
C3	1	1	1	1											
C4	0	0	0	0	1										
C5	1	1	1	1	2	1									
C6	0	0	0	0	1	0	1								
C7	1	1	1	1	2	1	2	1							
S1	0	0	0	0	1	0	1	0	1						
S2	1	1	1	1	2	1	2	1	2	1					
S3	1	1	1	1	2	1	2	1	2	1	2				
S4	2	2	2	2	3	2	3	2	3	2	3	3			
S5	0	0	0	0	1	0	1	0	1	0	1	1	2		
S6	1	1	1	1	2	1	2	1	2	1	2	2	3	1	

Nt. Position	123	456	789	111 012	111 345	111 678	122 901	222 234	222 567	223 890	333 123	333 456	333 789	444 012	444 345	444 678	455 901	555 234
Ser	TCA	ACA	AGA	GAT	GGC	GAA	CCC	CTC	ATG	ATA	GTG	GCA	AAA	CAT	GAA	AGG	GGG	AGA
C6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C7	...	...	...	...	...	...	...	...	...	...	...	...	G..	...	...	...	...	...
S1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S6	...	...	...	...	...	...	...	...	...	...	...	...	...	..C	...	...	...	...

	555 567	556 890	666 123	666 456	666 789	777 012	777 345	777 678	788 901	888 234	888 567	889 890	999 123	999 456	999 789	111 000 012	111 000 345	111 000 678
Ser	CCT	CTC	TTG	TTT	AAG	ACA	ACA	GAG	GGG	ATC	AAC	AAA	TGC	ACT	CTC	ATT	GCC	ATG
C6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S5	...	...	...	...	...	...	...	...	...	...	...	...	...	..C	...	...	...	...
S6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

Figure 4.3. Comparison of the sequence of a 282 nucleotide length coding region of prM gene from dengue-4 viruses. Ser= sequence that was RT-PCR amplified from serum. C6= sequence that was RT-PCR amplified from the same virus that was passaged two times in C6/36 cells. C1 to C7, sequence of cDNAs that were cloned from the cDNA amplified from the viruses passaged in C6/36 cells, S1 to S6 sequence of cDNAs amplified from the patients serum.

	111	111	111	111	111	111	111	111	111	111	111	111	111	111	111	111	111	111
	011	111	111	112	222	222	222	333	333	333	344	444	444	445	555	555	555	666
	901	234	567	890	123	456	789	012	345	678	901	234	567	890	123	456	789	012
Ser	GAC	TTG	GGT	GAA	ATG	TGT	GAG	GAC	ACT	GTC	ACG	TAT	AAA	TGC	CCC	CTA	CTG	GTC
C6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	G..	...
C6	...	...	...	...	...	...	...	...	G..	...	...	...	...	...	...	...	...	...
C7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..T	...	...	...
S5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

	111	111	111	111	111	111	111	111	111	111	111	111	122	222	222	222	222	222
	666	666	677	777	777	778	888	888	888	999	999	999	900	000	000	001	111	111
	345	678	901	234	567	890	123	456	789	012	345	678	901	234	567	890	123	456
Ser	AAT	ACC	GAA	CCT	GAA	GAC	ATT	GAT	TGC	TGG	TGC	AAC	CTC	ACG	TCT	ACC	TGG	GTC
C6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
C7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S3	...	...	...	...	...	...	...	...	...	...	C..	...	...	...	...	...	...	...
S4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S5	...	...	...	...	...	...	...	...	...	...	...	...	..T	...	...	...	...	...
S6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...

Figure 4.3. (CONTINUED)

```
     222 222 222 222 222 222 222 222 222 222 222 222 222 222 222 222 222 222
     111 222 222 222 233 333 333 334 444 444 444 555 555 555 566 666 666 667
     789 012 345 678 901 234 567 890 123 456 789 012 345 678 901 234 567 890 "

Ser  ATG TAT GGG ACA TGT ACC CAG AGC GGA GAA CGG AGA CGA GAG AAG CGC TCA GTA
C6   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
C1   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
C2   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
C3   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
C4   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
C5   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
C6   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
C7   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
S1   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
S2   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
S3   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
S4   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
S5   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...
S6   ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ...

     222 222 222 222
     777 777 777 888
     123 456 789 012

Ser  GCT TTA ACA CCA
C6   ... ... ... ...
C1   ... ... ... ...
C2   ... ... ... ...
C3   ... ... ... ...
C4   ... ... ... ...
C5   ... ... ... ...
C6   ... ... ... ...
C7   ... ... ... ...
S1   ... ... ... ...
S2   ... ... ... ...
S3   ... ... ... ...
S4   ... ... ... ...
S5   ... ... ... ...
S6   ... ... ... ...
```

Figure 4.3. (CONTINUED)

```
Amino          1 11111 11112 22222 22223 33333 33334 44444 44445 55555 55556
acid 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890 12345 67890
posi
tion

Ser  STRDG EPLMI VAKHE RGRPL LFKTT EGINK CTLIA MDLGE MCEDT VTYKC PLLVN TEPED
C6   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
C1   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
C2   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
C3   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
C4   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
C5   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..V.. .....
C6   ..... ..... ..... ..... ..... ..... ..... ..... ....A ..... ..... .....
C7   ..... ..... ..E.. ..... ..... ..... ..... ..... ..... ..... ..... .....
S1   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
S2   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
S3   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
S4   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
S5   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....
S6   ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... ..... .....


     66666 66667 77777 77778 88888 88889 9999
     12345 67890 12345 67890 12345 67890 1234

Ser IDCWC NLTST WVMYG TCTQS GERRR EKRSV ALTP
C6   ..... ..... ..... ..... ..... ..... ....
C1   ..... ..... ..... ..... ..... ..... ....
C2   ..... ..... ..... ..... ..... ..... ....
C3   ..... ..... ..... ..... ..... ..... ....
C4   ..... ..... ..... ..... ..... ..... ....
C5   ..... ..... ..... ..... ..... ..... ....
C6   ..... ..... ..... ..... ..... ..... ....
C7   ..... ..... ..... ..... ..... ..... ....
S1   ..... ..... ..... ..... ..... ..... ....
S2   ..... ..... ..... ..... ..... ..... ....
S3   ....R ..... ..... ..... ..... ..... ....
S4   ..... ..... ..... ..... ..... ..... ....
S5   ..... ..... ..... ..... ..... ..... ....
S6   ..... ..... ..... ..... ..... ..... ....
```

Figure 4.4. Deduced amino acid sequence of the 282 nucleotide length coding region listed in Figure 4.3.

DISCUSSION

The selection effect that different cell lines have on virus populations has been used to obtain viral variants. For example, vaccine production has been based in part on the selection of viral mutants obtained after serial passage of viruses in cell lines. Polivirus, a neurotropic virus, was attenuated by serial passage in primary monkey kidney cells at low temperature (Sabin, 1957). The viral sub-population that was selected using this procedure preserved its antigenicity, and the virus also replicated in epithelial cells of Peyer's patches in the intestinal tract of humans. However, it was no longer neurotropic. Nucleotide changes in the virus genome have been associated with passage in specific cell lines. For example, different mutations predominate depending upon the specific cell lines in which the viruses replicated (Rezapkin *et al.*, 1994). Even in the same cell line, the status of the cultured cells in which viruses replicate can influence the selection of virus mutants. Overgrown cells preferentially select for revertants of poliovirus compared with cells grow to normal confluence (Chumakov *et al.*, 1994). To develop a live attenuated dengue vaccine, dengue viruses have been passaged repeatedly in dog PDK cells. That unnatural cell environment exerts selective pressure on viruses, selecting for avirulent viruses (Bhamarapravati *et al.*, 1987; Bhamarapravati and Yoksan, 1997).

In the present study, 4 out of 5 (80%) nucleotide replacements in the clones obtained from amplification of viral RNA from serum were synonymous. However none of the nucleotide replacements in the clones obtained from amplication of viral RNA from C6/36 cells were synonymous; all 3 nucleotide substitutions were non-synonymous. The bias for either synonymous or non-synonymous substitutions indicates purifying and positive

selection in serum and cells, respectively. The presence of non-synonymous mutations in those analyzed clones from viruses passaged in C6/36 cells suggests the existence of variants with amino acid changes. In addition, the existence of synonymous mutations in several of the clones from viruses from serum highlights the variability of the RNA genome. RNA viruses, even those initiated from a single infectious unit, consist of a large number of genetic microvariants (Eigen and Biebricher, 1988; Holland *et al.*, 1992). The high genetic variability in these virus populations can facilitate the rapid adaptation to new microenvironments. The results found in this study suggest that dengue viruses are composed of a number of genetic variants. It is possible that passage of dengue viruses in the cell culture micro-environment selects for viral variants of the quasispecies that are more fit in the new micro-environment. The procedure used in these experiments, SSCP and nucleotide sequencing of cDNAs RT-PCR amplified from human serum and C6/36 cells, and SSCP and nucleotide sequencing of cDNAs that were cloned from the RT-PCR products, provided information about the composition and abundance of virus haplotypes in the clinical sample analyzed and of the consequences of dengue virus passage in the two microenvironments. Clearly, dengue viruses, as other RNA viruses have considerable evolutionary potential and can readily adapt to new microenvironment. However, it is also possible that nucleotide sequence errors introduced by the RT-PCR assay, had resulted those variants detected by SSCP. The preliminary results reported here should be confirmed, analyzing a greater number of clones.

Chapter 5

DENGUE VIRUS SUB-POPULATIONS ASSOCIATED WITH DIFFERENT DISEASE OUTCOMES

INTRODUCTION

Human infections with dengue viruses may be asymptomatic. However, masive epidemics can occur, resulting in high number of symptomatic cases. When symptomatic, dengue infection can result in undiferentiated fever, dengue fever (DF) and dengue hemorrhagic fever (DHF) (Burke *et al.*, 1988; Innis, 1995; Gubler, 1998). DF is self-limiting and the symptoms are often mild. Common symptoms are fever, cephalalgia, myalgia, arthralgia, retro-orbital pain, and in some instances rash. DHF has many of the same clinical manifestations as DF in addition to hemorrhage. In this more severe form of the disease, there can also be plasma leakage to the extravascular compartment and a drastic decline in the number of circulating platelets. Plasma leakage, is considered the pathognomonic feature of the DHF syndrome, but hemorrhage can also occur (Petersdorf *et al.*, 1983; Kalayanarooj *et al.*, 1997). Other clinical manifestations of dengue disease have been reported, including hepatitis, cardiomyopathy and encephalopathy (Lum *et al.*, 1996; Nimmannitya *et al.*, 1987; Mehendale *et al.*, 1989; Lum *et al.*, 1996; Row *et al.*, 1996; Hommel *et al.*, 1998; Couvelard *et al.*, 1999)). However, these clinical forms are rare.

Two principal biological mechanisms (immune enhancement and more virulent viruses) have been hypothesized to condition the disease severity that is observed in patients with DHF. In the first mechanism, prior infection with a dengue serotype predisposes individuals to more severe disease when infected with a second and different serotype. The first infection produces antibodies that cross react with heterologous dengue virus serotypes, but do not neutralize (Sangkawibha *et al.*, 1984; Burke *et al.*, 1988). This presumably facilitates the viral entry into monocytes by opsonization, and these cells

protect the opsonized virus from the potentially hostile components of the immune system (Halstead, 1988). The second hypothesis is that certain genetic strains of dengue virus are more virulent and can cause a more severe disease (Gubler, 1988). The two mechanisms are not mutually exclusive.

Viruses with RNA genomes exist as populations of closely related but distinct genotypes: the quasispecies (Eigen and Biebricher, 1988). Thus viruses existing in nature are not a group of identical copies of the same genome, but a population composed of multiple closely related sub-populations (Eigen and Biebricher, 1988). Based on this perspective, DHF may occur when a viral variant with higher virulence infects a susceptible host. The severity of the clinical manifestations occurring during the disease caused by dengue virus could be the result of the existence of viral sub-population with increased virulence.

Because there is no animal model for DF and DHF, support for these hypotheses is based on epidemiologic observations in endemic areas (Halstead *et al.*, 1970). More than 90% of the cases of DHF occur in patients with antibodies to heterotypic dengue virus infections. In addition, it has been suggested that maternal antibodies to dengue virus protect infants against DHF during the first months of age when neutralizing activity is high. However, the same antibodies can increase the risk of the disease when neutralization activity decreases (Kliks *et al.*, 1988). These observations provide support for the immune enhancement hypothesis (Halstead *et al.*, 1970, 1973). On the other hand, DHF cases have been reported in regions where dengue viruses have been introduced for the first time or after many years of absence (Barnes and Rosen, 1974). In those cases, the

patients had primary infections, and no antibodies against previous dengue virus could be detected (Barnes and Rosen, 1974; Scott *et al.*, 1976; Morens *et al.*, 1987). These observations provide support for the virulent virus genotype hypothesis.

Other observation also support the second hypothesis. The rapid increase in the incidence of DHF in some geographic regions also may be due to the existence of viral strains with different transmission potential (Gubler *et al.*, 1978). In addition, the virus isolation rate obtained from the serum of patients from different geographic areas indicates the possibility of virus strains capable of causing higher viremias than other strains of the same dengue virus serotype. Particularly interesting has been the observation that in those areas where the isolation rate was high, DHF disease was more severe (Gubler, 1997).

Epidemiological studies suggest that there are qualitative differences in the severity of the disease associated with some viral strains. Dengue-2 viruses that have historically circulated in the Caribbean and several countries in Latin America cause a milder disease than dengue-2 viruses that caused the epidemic in Cuba during 1981. In that epidemic, there were 34,000 cases of DHF occurring in a 5 month period (Kouri *et al.*, 1986). A dengue-2 virus that was isolated in Jamaica in 1983 (Jamaica topotype) was assumed to be the same virus transmitted in Cuba during 1981. Dengue-2 viruses (Puerto Rico topotype) circulated in the Caribbean previous to 1981. The Puerto Rican and Jamaican topotypes were clearly different by RNase T1 fingerprinting analysis (Trent *et al.*, 1983, 1990) The Jamaican virus was genetically related to dengue-2 viruses isolated in Thailand in 1980 and Vietnam in 1987 (Rico-Hesse, 1990). Thus the virus that caused the Cuban

epidemic presumably had an Asian origin. Nucleotide sequence analysis of viruses isolated during epidemics in Brazil and Venezuela, in which DHF cases occurred, supported the idea that the viruses from all three epidemics belonged to the same genotype (Lewis *et al.*, 1993).

Other epidemiologic observations suggest genetic polymorphism in viruses could account for epidemic potential and virulence. Dengue-2 virus transmission occurred on the islands of Fiji, Tahiti, New Caledonia and Niue Island in the South Pacific during 1971 and 1972. These epidemics were referred to as "explosive" outbreaks (Gubler, 1988). The incidence of disease from this epidemic was high and severe, particularly in Niue Island (Loison *et al.*, 1973; Moreau *et al.*, 1973; Barnes and Rosen, 1974; Maguire *et al.*, 1974). In contrast, during 1972, an epidemic associated with dengue-2 virus occurred in American Samoa. This epidemic caused a milder form of disease than that which occurred in the other islands (Gubler, 1988). A year later, dengue-2 virus was detected in Tonga. Dengue-2 virus circulated for a year on this island, but went almost undetected by the medical community (Gubler *et al.*, 1978).

Studies were conducted to test the hypothesis that genotypic differences in the viruses could condition DF or DHF. Previous results (Chapter 2) clearly demonstrated that SSCP analysis can differentiate closely related genomic sequences among dengue viruses. In these studies viral isolates from patients with DF were compared with those obtained from patients with DHF. The prM region of 8 dengue-2 viruses were characterized by SSCP.The hypothesis tested was that the viral populations in DF or DHF patients, are composed of a mixture of viruses with related but diverse nucleotide sequences in their

genomes, and that some viral subpopulation(s) were present only in patients with DHF. The distribution of the genomic variants in the populations of dengue viruses were determined. The goal was to elucidate possible dengue viral markers of disease severity, that could be identified by SSCP analysis.

MATERIAL AND METHODS

Specimens

Eight dengue-2 virus isolates were analyzed in this study. Viruses were obtained from mosquitoes that were intrathoracically inoculated with sera from patients that were collected during the 1975 and 1976 epidemics in Indonesia and were kindly provided by Dr. Duane J. Gubler from the Centers for Disease Control and Prevention) (Eram *et al.*, 1979). The serotype of the viruses were identified by the complement fixation test using viral antigens obtained from infected mosquitoes (Gubler *et al.*, 1979b). The pertinent information concerning the isolates is presented in Table 5.1.

ID	Place	Year	Disease outcome
BC40/97	Jakarta	1975	DF
BC42/97	Jakarta	1975	DF
BC43/97	Jakarta	1975	DF
BC52/97	Jakarta	1975	DF
BC47/97	Jakarta	1976	DHF IV
BC48/97	Jakarta	1976	DHF III
BC53/97	Jakarta	1975	DHF III
BC54/97	Jakarta	1975	DHF IV

Table 5.1 Epidemiological and clinical information of dengue-2 isolates. All the isolates had been passed once in mosquitoes and once in C6/36 cells.

Mosquito processing

Mosquitoes infected by intrathoracic inoculation with serum samples from Indonesia had been stored since the late 1970's at -70°C. One or two infected mosquitoes of each sample were triturated in 1.0 ml of medium M-199 containing 5% heat-inactivated fetal bovine serum (FBS) and 100 U/ml of penicillin, 100 µg/ml of streptomycin, 1µg/ml of fungizone and 50 µg/ml of gentamicin sulfate. Suspensions were centrifuged 1 min at 8,000 rpm, filtered through 0.45 µm pore filters, and stored at -70°C until use.

Virus isolation

Dengue viruses were isolated using the C6/36 clone of *Aedes albopictus* mosquito cell line. Confluent monolayers of the C6/36 cells were grown in 25 cm^2 flasks for 3 days at 28°C. Dulbecco's modified Eagle's growth medium (DMEM) was used which contained 10% heat-inactivated fetal bovine serum (FBS), and was supplemented with 0.01 mM non-essential amino acids, 0.01 mM of sodium pyruvate, and 0.9 mg of sodium bicarbonate, 100 U of penicillin, 100 µg of streptomycin and 1 µg of fungizone per ml. To infect the cells, the growth medium was replaced with 1 ml of maintenance medium, (DMEM containing 2% FBS instead of 10%). Thirty µl of each specimen (serum or mosquito triturate) was added to the cells and incubated at 28°C. After an adsorption period of 1 hour, 7 ml of maintenance medium was added to each flask. The flasks were incubated at 28°C for 15 days. At day 7 post infection (pi), the medium was replaced with fresh medium. The infected cells were harvested on days 10 and 15 (pi) and pelleted by centrifugation. The cells were then resuspended in PBS, pH 7.2. Twelve well spot slides were prepared using 15 µl of cell suspension per spot. The slides were air dried, fixed in

cold acetone (-20°C) for 15 min and analyzed by immunofluorescence immediately or stored at –70°C until use. The serotype of each virus was determined by immunofluorescence analysis using serotype specific antibodies (Henchal *et al.*, 1982, 1983).

RNA extraction and RT-PCR synthesis of cDNA

RNA extractions were conducted with the infected cell suspension from the eight specimens under study and cDNA was synthesized for each. PCR was done with cDNA obtained from the eight specimens. The procedures used for RNA extraction and PCR amplification were described in Chapter 3. The primers that were used in the reaction, length of the DNA amplified, and annealing temperature are listed in Table 5.2.

Primer ID	Sequence	Prod. Length (nt)	Ann. Temp (°C)
dn2prm1**f**	5'-ACCACACGTAACGGAGAACCA-3'	291	54.7
dn2prm1**r**	5'-TCCCACATGTGGAACGAGTGC-3'		

Table 5.2. Primers used for the SSCP analysis of the dengue virus isolates (**f**=forward primer, **r**=reverse primer). The size of the RT-PCR products are presented.

SSCP analysis

SSCP analyses were performed as described in Chapter 3. cDNA was obtained by RT-PCR from each of the 4 patients with DF and DHF, respectively. In addition to the cDNA obtained from the viral samples, DNA was PCR amplified from bacterial colonies

after cloning and analyzed by SSCP.

Cloning of RT-PCR products

cDNAs obtained from each one of the RT-PCR amplifications of the eight virus isolates were cloned using the procedure described in Chapter 4. Bacterial colonies were screened by PCR using the same primers that were used for the RT-PCR procedure and shown in (Table 5.2). The procedure for prM amplification, cloning, and SSCP analysis is presented in Figures 5.1A and 5.1B.

Statistical analysis

The diversity of the banding patterns (SSCP haplotypes) was analyzed using the Keefe and Bergersen's diversity index T (Keefe and Bergersen, 1977) where: $T= 1- \Sigma (p_i)^2$, and p_i is the proportion of the haplotype i among all haplotypes in the group. For this analysis, the unbiased estimator $TU=1(n/(n-1))\{ \Sigma (p_i)^2-(1/n)\}$ was used. The diversity among a) SSCP haplotypes comprising a viral isolate from each patient, regardless of the disease outcome, and b) SSCP haplotypes comprising an isolate from patients in each one of the two disease outcomes, were estimated. To assess the significance of the difference between two diversity indexes, the Z test value was calculated ($Z=(TU1-TU2)/\{(\sigma_1)^2/n_1+(\sigma_2)^2/n_2\}^{-1}$).

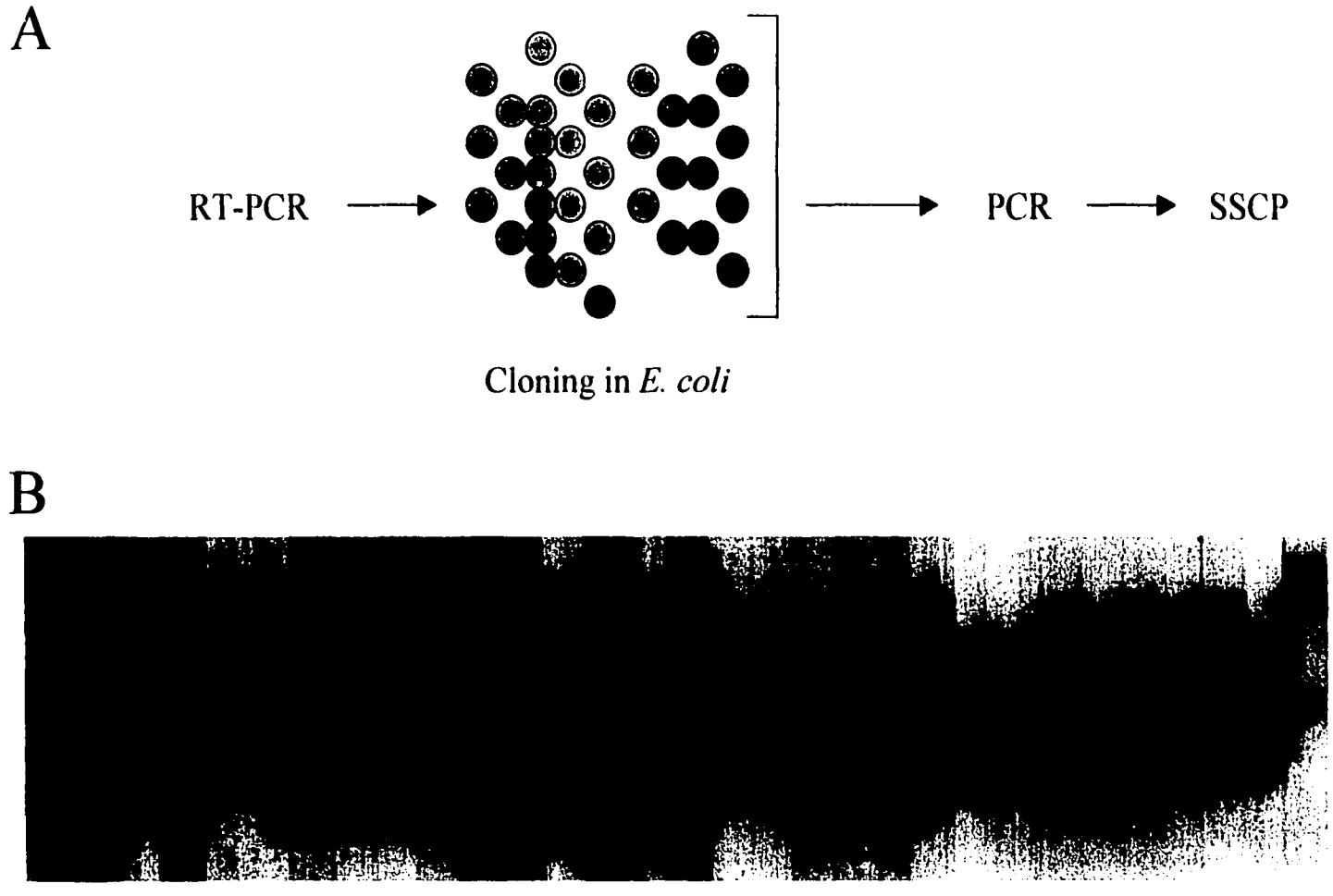


Figure 5.1. A. Protocol used to assess the presence of genomic diversity in the population of viruses present in C6/36 cells and **B.** SSCP gel showing the diversity of the banding patterns obtained from different clones.

RESULTS

Analysis of clonal DNA by SSCP

cDNA fragments that correspond to 291nt sequence from the prM coding region were obtained by RT-PCR. SSCP analysis of the DNAs revealed seven different banding patterns. Only two (BC47 and BC48) out of eight specimens gave similar banding patterns (Figure 5.2). Therefore it was not possible to associate a specific banding pattern with disease outcome.

In order to rule out the possibility that the diversity in the SSCP patterns was the result of artifacts introduced during the cloning procedure, DNA was amplified by PCR from one of the clones derived from the BC47/97 dengue-2 virus isolate, which was cloned again following the same procedure used for the initial cloning event (Figure 5.3A). Thirty clones were amplified by PCR and the cDNAs analyzed by SSCP. Identical SSCP patterns were found in the products of all clones (Figure 5.3B).

The complexity of the viral sub-populations present in each one of the specimens was assessed as a possible indicator of disease outcome. From each one of the 4 patients with DF, the SSCP patterns from 44, 46, 43 and 44 clones were analyzed . SSCP analysis was also performed on 60, 65, 40 and 63 clones derived from the 4 patients with DHF. For each one of the 8 specimens, one SSCP banding pattern that was predominant among the clones was identified, as well as several different patterns that were present in a more reduced number. The complete list of the number of clones analyzed and SSCP genotypes that were identified in each patient are presented in Table 5.3. The SSCP genotypes obtained with the 8 isolates are presented In Figures 5.4, 5.5, and 5.6.

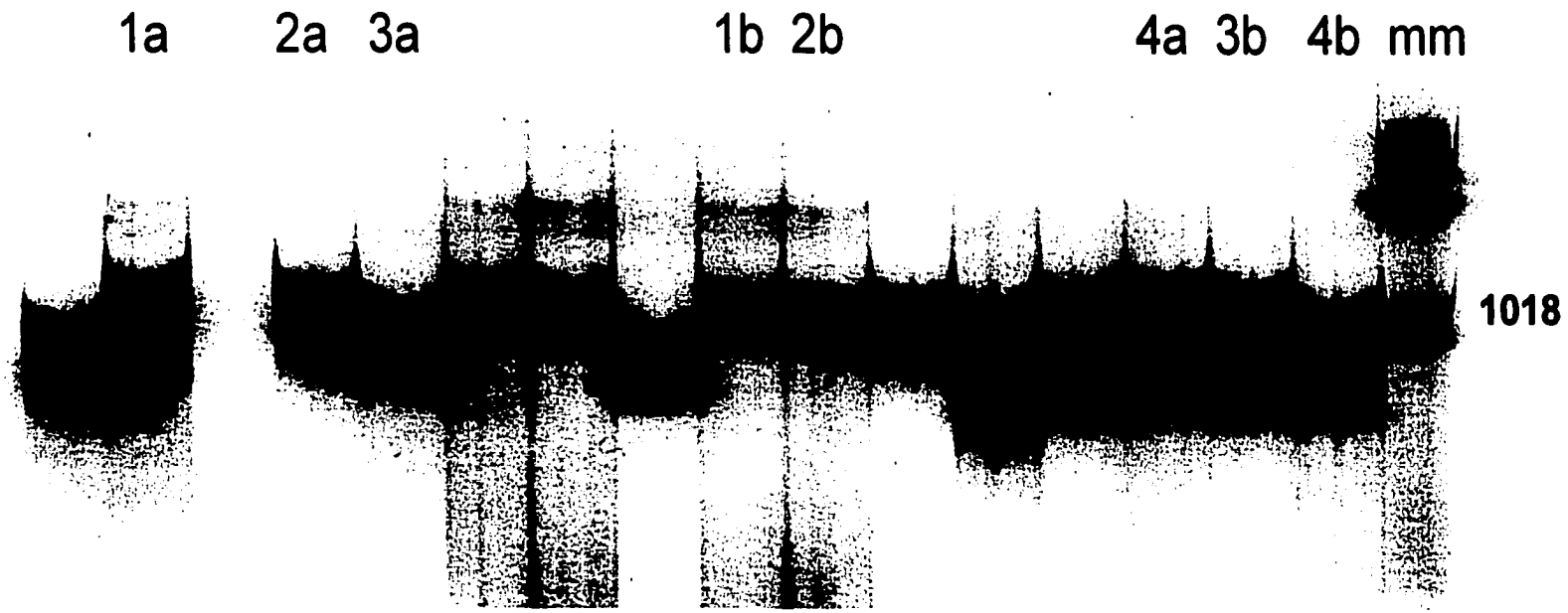
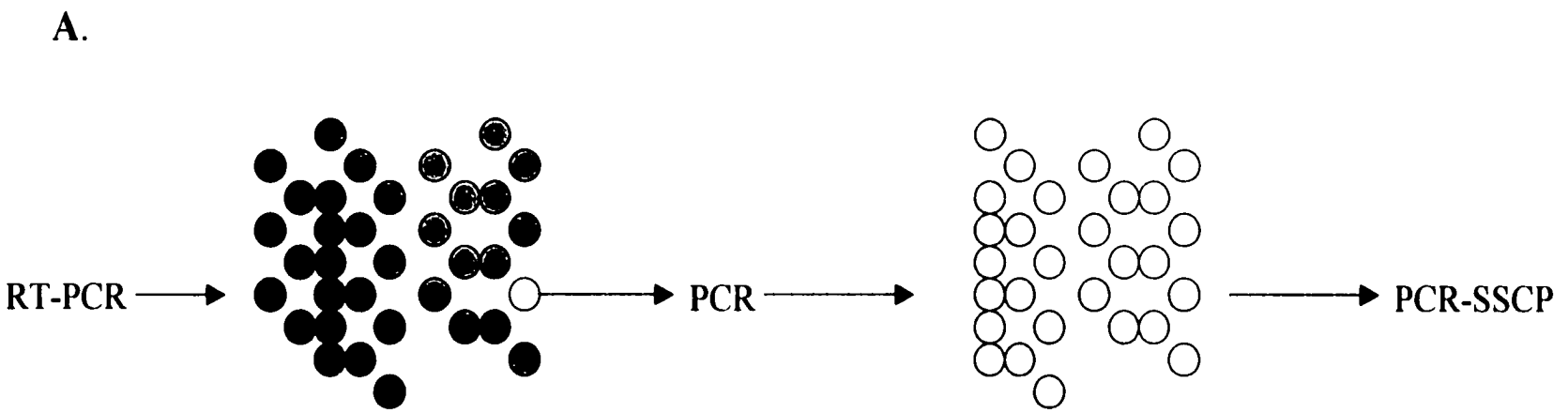


Figure 5.2. SSCP analysis of DF and DHF viruses from Jakarta 1975-1976. 1a=BC40, 2a=BC42 3a=BC43, 4a=BC52, 1b=BC47, 2b=BC48, 3b=BC53, 4b=BC54. The four specimens with ID ending with a are from patients with DF diagnosis, those ending with b are from DHF



B. PCR-SSCP analysis of DNA of clones from a single clone

Figure 5.3. **A.** Scheme of the cloning procedure used to preclude the possibility of introduction of cloning artifacts. **B.** SSCP bands obtained from the cloning of the PCR product amplified from a clone of isolate BC47/97.

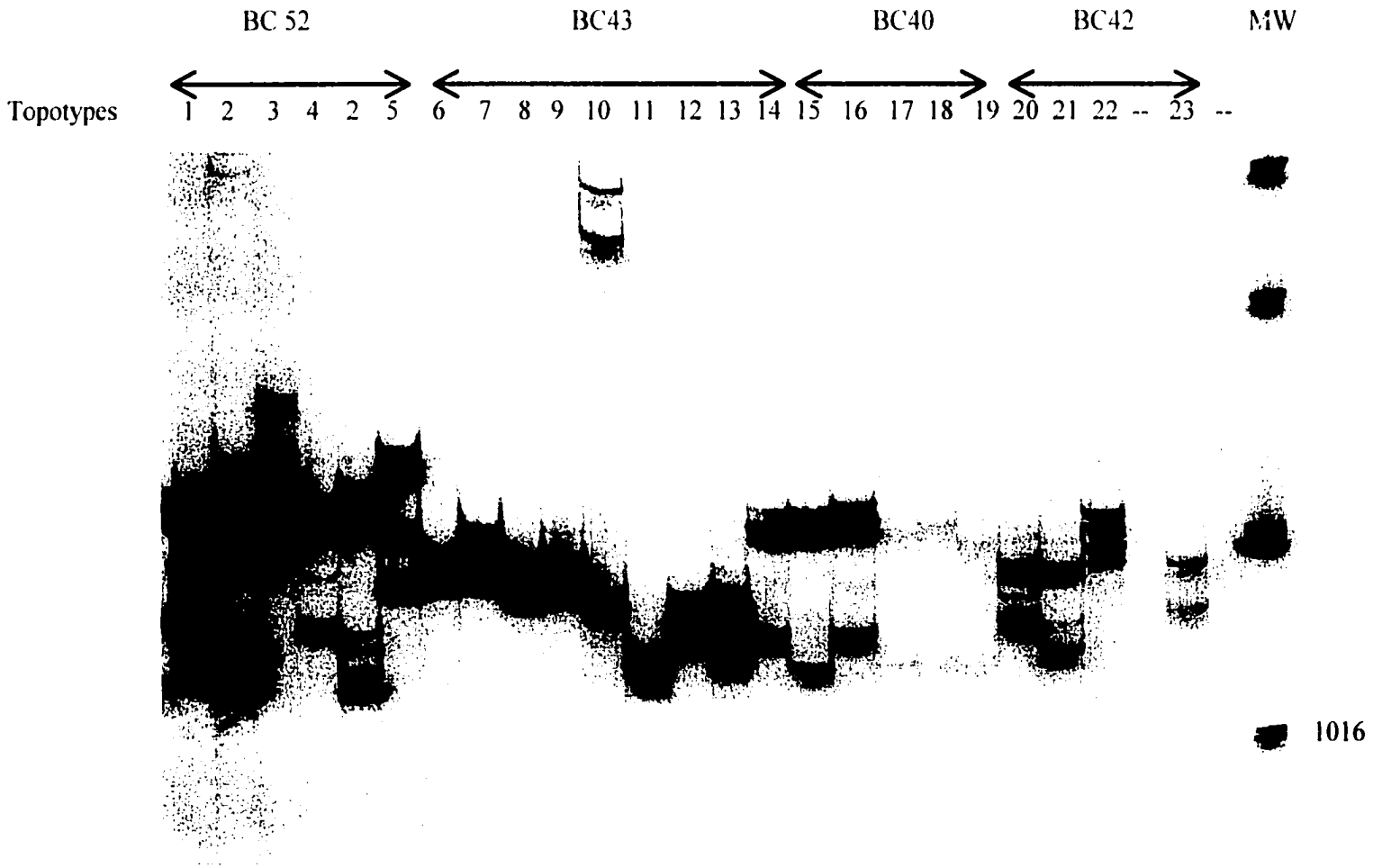


Figure 5.4. SSCP analysis of DNA from clones of cDNA sequences amplified from isolates of four DF cases (BC52, BC43, BC40 and BC42). For each isolate, one haplotype predominates. The haplotype numbers correspond to those shown in Table 5.3.

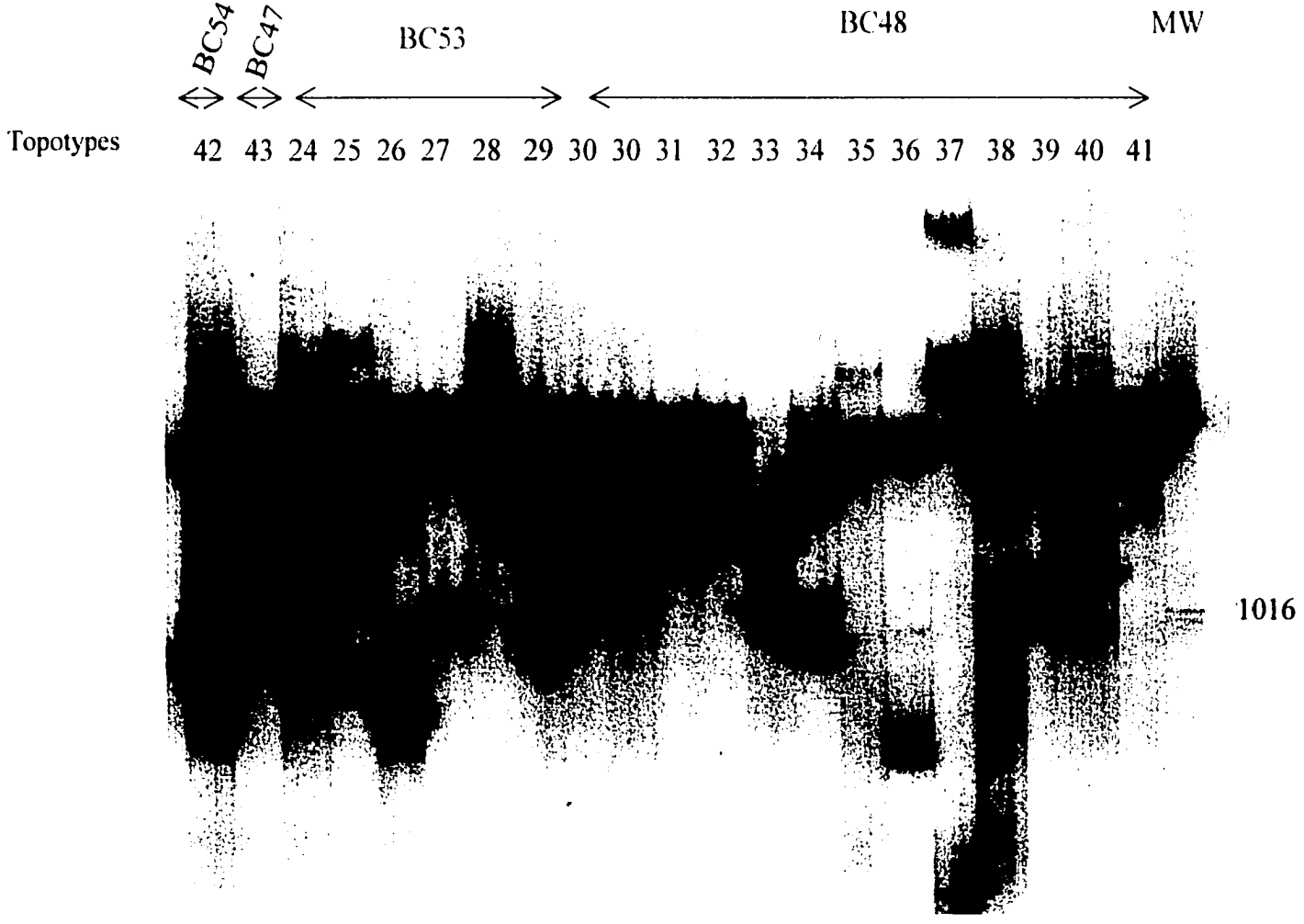


Figure 5.5. SSCP analysis of DNA from clones of cDNA sequences amplified from isolates of two DHF cases (BC53 and BC48). For each isolate, one haplotype predominates. (One of the majority haplotypes for the other DHF isolates (BC54 and BC47) are also presented). The haplotype numbers correspond to those shown in Table 5.3.

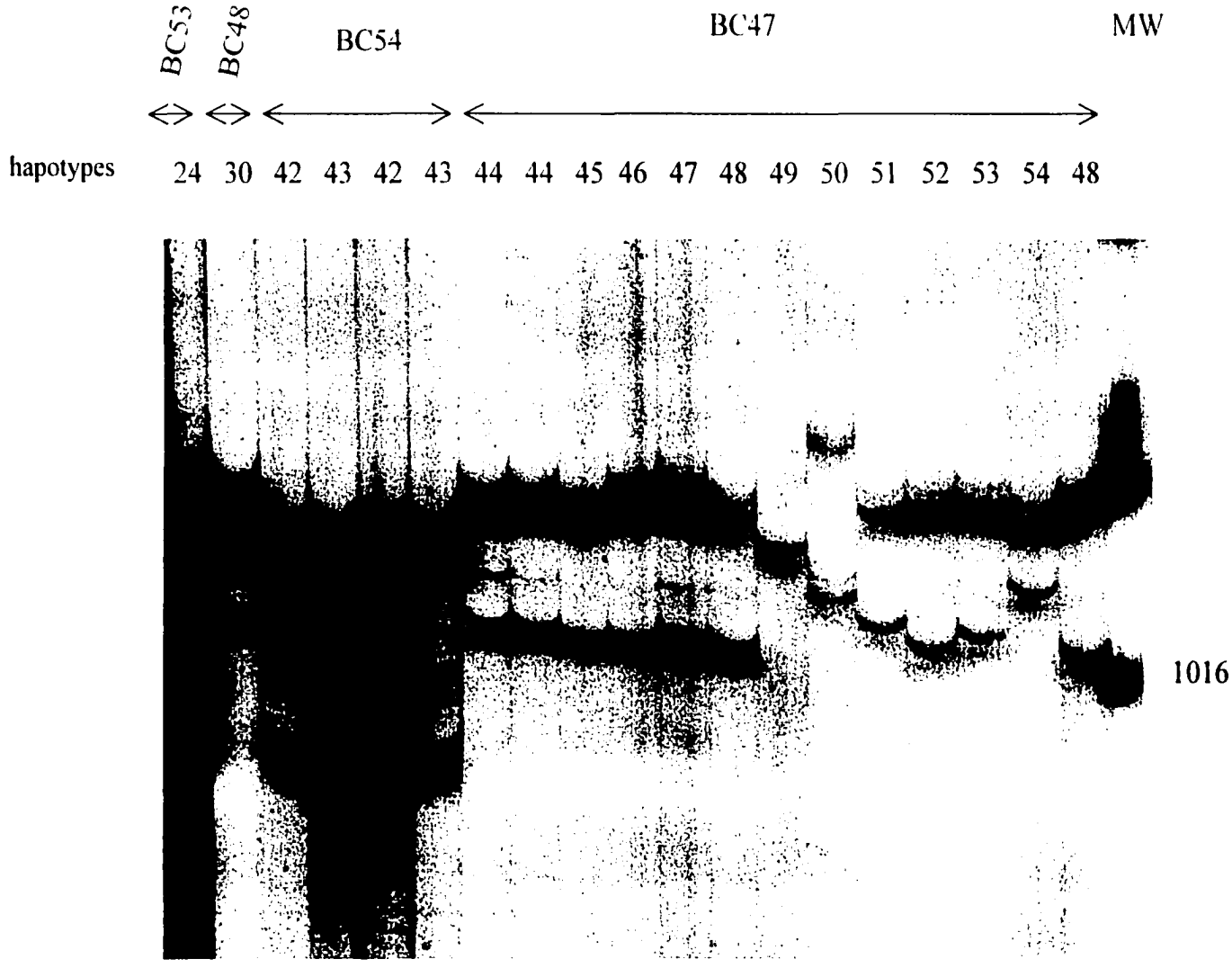


Figure 5.6. SSCP analysis of DNA from clones of cDNA sequences amplified from isolates of two DHF cases (BC54 and BC47). For each isolate, one haplotype predominates. One of the majority genotypes for the other DHF isolates (BC53 and BC48) are also presented. The haplotype numbers correspond to those shown in Table 5.3.

	Specimen ID	Disease outcome	Haplotype number (number of identical components in each haplotype)	Number of haplotypes	Number of clones analyzed
1	BC52	DF	1(39), 2(2), 3(1), 4(1), 5(1)	5	44
2	BC43	DF	6(38), 7(1), 8(1), 9(1), 10(1), 11(1), 12(1), 13.(1) 14(1)	9	46
3	BC40	DF	15(39), 16(1), 17(1), 18(1), 19(1)	5	43
4	BC42	DF	20(41), 21(1), 22(1), 23(1)	4	44
5	BC53	DHF	24(55), 25(1), 26(1), 27(1), 29(1), 29(1)	6	60
6	BC48	DHF	30(54), 31(1), 32(1), 33(1), 34(1), 35(1), 36(1), 37(1), 38(1), 39(1), 40(1), 41(1)	12	65
7	BC54	DHF	42(38), 43(2)	2	40
8	BC47	DHF	44(52), 45(1), 46(1), 47(1), 48(2), 49(1). 50(1), 51(1), 52(1), 53(1), 54(1)	11	63

Table 5.3 Analysis of dengue-2 haplotypes from DF and DHF patients.

Analysis of SSCP haplotypes diversity in dengue fever and dengue hemorrhagic fever patients

To analyze the diversity of SSCP haplotypes in DF and DHF patients, the Keefe and Bergeson diversity index (TU) was calculated for the haplotypes for each virus isolate and compared between DF and DHF patients (Table 5.4). A paired comparison of the TU corresponding to the haplotypes in each isolate from the 8 patients indicated significant differences between patients 2 (BC43) vs. patient 7 (BC54), 6 (BC48) vs. 7 (BC54) and 7 (BC54) vs. 8 (BC47). All the other paired comparisons between patients did not result in significant differences.

Clinical Outcome (Disease)	Patient number	Diversity in each patient	Diversity in Outcome (Disease)
DF			0.8070
	1	0.2156	
	2	0.3208	
	3	0.1794	
	4	0.1332	
DHF			0.8088
	5	0.1610	
	6	0.3739	
	7	0.0974	
	8	0.3205	

Table 5.4 Keefe and Bergeson analysis (TU) of SSCP pattern diversity in patients and disease outcome.

In each one of the patients, a proportion of the haplotypes differed from the predominant one. The proportions of haplotypes from isolates from DF patients differing from the corresponding majority haplotype were: 0.114, 0.174, 0.093, and 0.068, respectively. In those with DHF, the proportions were 0.083, 0.169, 0.05, and 0.175. In each group of 4, the proportions were tested to see if they differed from the others. The isolates from DF and DHF patients were tested separately. The proportions of the less abundant haplotypes did not differ statistically in isolates from patients with DF (Chi square=2.76, D.F.=3, p=0.4307). Similarly the isolates from DHF patients did not differ (Chi square=5.50, D.F.=3, p=0.1388). The proportion of the minor SSCP haplotypes in the 4

isolates from DF patients did not statistically differ from the proportion of the minor genotypes in the isolates from DHF patients . The proportions were 0.113 and 0.127 for the isolates from patients with DF and DHF, respectively. There was no significant difference between the proportions (Chi square=0.19, D.F.=3, p=0.6638).

DISCUSSION

Several attempts have been made to find an association between specific dengue virus genotypes and increased virulence. Virulence of a virus is defined as "the capacity of a virus, compared to other closely related viruses, to produce disease in a host" (Tyler and Fields, 1996).

The SSCP analysis demonstrated diversity in virus populations in the patients with DF and DHF. In each of the dengue-2 isolates, a number of viral variants were present. Each one of these variants from 125 haplotypes could be readily identified by its distinct SSCP banding pattern. Genetic variability within virus populations derived from dengue-2 viral isolates from patients with DF and DHF was demonstrated, even though the 291 nt prM sequence analyzed represented less that 3% of the total length of the viral genome.

In all SSCP analyses, one of the haplotypes within a virus population predominated and other haplotypes were present in lower proportions. All the haplotypes identified were different. The SSCP procedure does have limited resolution and it is difficult to compare SSCP patterns when the bands do not differ significantly in migration in the polyacrylamide gel. When the eight dengue-2 virus isolates were analyzed by SSCP before the cloning procedure, seven different banding patterns were identified. Only isolates BC47/97 and BC48/97 gave banding patterns that looked identical by SSCP (Figure 5.2, 1b and 2b).

However, after the cloning procedure, the haplotypes that predominated in the two viral isolates (BC47/97 and BC48/97) looked similar, but not identical (Figure 5.6 haplotypes 30 and 44). Variability in the 291 nt fragment has been reported in another group of eight dengue-2 isolates, which were sequenced from patients displaying different dengue disease severity (Mangada and Igarashi, 1998). Paired comparisons of the nucleotide sequences revealed 1 to 5 nucleotide replacements in that area of the viral genome (Mangada and Igarashi, 1998).

The results we found do not correlate differences in the prM SSCP haplotypes with differences in the severity of the disease. However, it is important to point out that the viruses used in this study had been passaged in mosquitoes. It is important to do a similar analysis with viruses obtained directly from patient's serum to prevent the possible selection effect of the passage in mosquitoes. However, the results do demonstrate that RT-PCR SSCP analysis can be used to identify haplotypic diversity of viruses that are present in the patient serum.

CHAPTER 6

SUMMARY

This dissertation research focused on the rapid detection and demonstration of the genomic variability of dengue viruses. The basic hypothesis was that each serotype of dengue virus would be comprised of multiple haplotypes. The haplotypes could be exploited for surveillance and for assessing virulence potential. It was also hypothesized that even single dengue viral isolates would be composed of a mixture of viruses with closely related genomes. Passage of these quasispecies in different mico-enviroments would result in preferential replication of different sub-populations or variants of the virus. A corollary of the last hypothesis was that specific subpopulations of dengue viruses could be associated with disease outcome, eg – dengue fever or DHF-SS.

A rapid and relatively inexpensive RT-PCR SSCP technique was developed and standardized to detect haplotypes in dengue virus isolates. Three possible genomic regions (E, prM, and NS5) were investigated as biomarkers for genetic polymorphisms in the genome, and a fragment of the gene coding for prM protein was selected as being the most informative.

The utility of the RT-PCR SSCP technique for dengue virus surveillance was investigated. A collection of the virus isolates from the Yucatan Peninsula of Mexico that included the four serotypes was characterized by RT-PCR SSCP. Each serotype was demonstrated to be compriised of multiple haplotypes. Six distinct dengue-1 SSCP

haplotypes were identified from the 23 virus isolates obtained from the Yucatán Peninsula, 3 dengue-2 haplotypes from 4 virus isolates, 4 dengue-3 haplotypes from 62 viral isolates, and 8 dengue-4 haplotypes from 45 viral isolates. Interestingly, limited haplotype diversity was found in dengue-3, the virus serotype more recently introduced into Mexico. These characterized viruses now provide an invaluable data base of dengue virus haplotypes to use for surveillance for introduction of new haplotypes into the region.

The effect of virus passage on dengue genetic diversity was explored. An RT-PCR, cloning and sequencing approach revealed that the nucleotide diversity of cDNAs amplified directly from virus from sera was predominantly due to synonymous substitutions. In contrast cDNAs amplified from viruses which had been passed in C6/36 cell culture had predominantly non-synonymous nucleotide substitutions. This strongly suggests that passage of dengue viruses in cell cultures rapidly selects for virus variants in the quasispecies that are more fit in the new micro-environment of the cell culture.

Since passage of viruses in cell culture rapidly selects the more fit in cell culture, virus variants associated with other phenotypes such as dengue disease severity could be missed in conventional laboratory bioassays. To investigate the possibility of a particular sub-population of a dengue virus quasispecies in an infected human could be associated with DHF, the composition of the virus populations isolated from patients with DF and DHF was analyzed, using RT-PCR, cloning and SSCP. Although the existence of genomic diversity was demonstrated in each one of the virus isolates analyzed, no particular subpopulation was correlated with the disease outcomes. However, it is important to note that the viruses analyzed had been passaged in cell culture already. Similar studies

comparing dengue virus diversity and haplotypes directly in patient specimens could be much more revealing in terms of associating virus variants with disease outcomes.

Dengue viruses do exhibit considerable genetic variability in nature. RT-PCR SSCP analysis of dengue viruses is clearly an advance which permits rapid and inexpensive surveillance and characterization of dengue viruses. Application of this technique in surveillance programs can provide rapid identification of newly introduced viruses, can provide information on viruses that have greater virulence potential and thus pose greater risk to human populations, and thus can permit public health authorities in resource limited areas to focus control efforts on areas with greater need. In addition, application of the technique with viruses amplified directly from clinical specimens may reveal genetic determinants of dengue virus virulence and thus may provide greater understanding of the pathogenesis of this very important public health pathogen in its human host.

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