CHAPTER III

LITERATURE REVIEW

I. Dengue Virus

1. Dengue Virus

The dengue virus are RNA viruses belonging to the genus *Flavivirus* of the *Flaviviridae* family, and their main arthropod vectors. The four serotypes of dengue virus (designated DEN-1, DEN-2, DEN-3, and DEN-4) can be distinguished by serological methods, plaque reduction neutralization test (3, 4, 8, 14). Infection in human by one serotype produces life-long immunity against reinfection by that same serotype, but only temporary and partial protection against the other serotypes (8). Similar to other *Flaviviruses*, dengue virus genome consist of a single stranded, positive-sense RNA of approximately 11 kb in length containing a single open reading frame. The mature dengue virion genome is surrounded by an icosahedral nucleocapsid about 30 nm in diameter. The nucleocapsid is covered by a lipid envelope about 10 nm. The complete virion is about 50 nm in diameter (14, 22, 49, 74). The virus genome is composed of three structural protein genes, encoding nucleocapsid or core protein (C), a membrane-associated protein (M), an envelop protein (E), and seven non-structural (NS) protein genes. The order of proteins encodes is 5'-C-prM (M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' (14, 47) (Figure 1). The structural proteins are included in the mature virion, whereas the non-structural proteins play various roles in virus replication and polypeptide processing (14) (Table 1). The individual virus proteins are cleaved after translation of entire polyprotein, which occurs at the rough endoplasmic reticulum (ER).

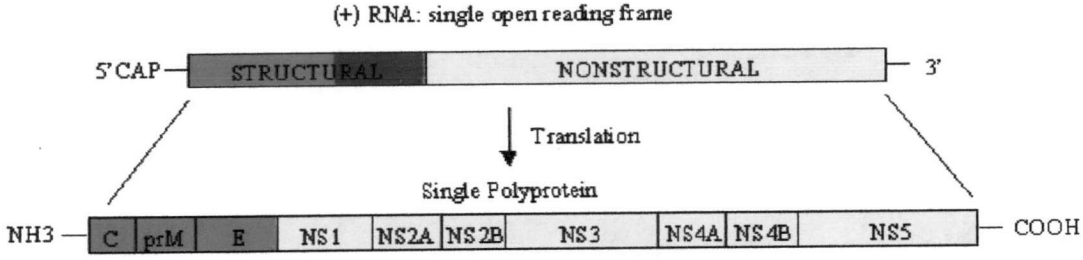

Figure 1 Dengue virus genome

Cell-associated virions within the ER are morphologically identical to extracellular particles. The C protein interacts with RNA to form the virion nucleocapsid. The prM (precursor of M) glycoprotein forms an intracellular heterodimer, stabilizing the E polypeptide during exocytosis. The prM protein is cleaved before virus release from the cell, leaving a small M structural protein anchored in the virus envelop, and releasing the larger 'pr' segment into the extracellular medium. The E glycoprotein contains antigenic determinants for hemagglutination and neutralization. Antibodies directed at E determinants also mediate the phenomenon of antibody-dependent enhancement (ADE) of infection in the pathogenesis of dengue hemorrhagic fever. The protein is also involved in attachment to cells and membrane fusion, and thus is a major factor in virus virulence (4, 7, 10, 48)

2. The Vector

The principal mosquito vector is *Aedes aegypti*, a tropical and subtropical species that was found around the world, usually between latitudes 35°N and 35°S, approximately corresponding to a winter isotherm of 10°C. *Aedes aegypti* has been found as far north as 45°N, such invasion has occurred during the warm season, and the mosquitoes have not survived the winters. Distribution of *Aedes aegypti* is also limited by altitude.

Table 1 Dengue proteins and functions

Protein		Function	
		Replication	Immunity
Structural	C	nucleocapsid structural/assembly	T-cell epitope
		stabilizes/regulates polymerization	protection
	PrM/M	of E protein	(neutralization)
		major enevelope protein, receptor	protection
	E	binding,	(neutralization)
		membrane fusion,	
		hemagglutination	CTL target
Nonstructural	NS1	soluble complement fixing antigen	CTL.ADCC
		membrane-bound glycoprotein	
	NS2A	virus-specific protease	CTL
	NS2B	unknown	
		protease, RNA helicase,	
	NS3	triphosphatase	CTL
	NS4A	replicase function	
	NS4B	replicase function	
		RNA polymerase,	
	NS5	methyltransferase	

It is usually found above 1000 m but has been reported at 2121 m in India, at 2200 m in Columbia, where the mean annual temperature is 17°C. *Aedes aegypti* is one of the most efficient mosquito vectors for arboviruses, because it is highly anthropophilic and thrives in close proximity to humans and often lives indoor. *Aedes aegypti* breeds in artificial containers with clean water in and around human habitations and bites principally during the daytime. This species became highly adapted to living in intimate association with humans and highly efficient epidemic vector in urban setting. Secondary vectors include *Aedes albopictus*, *Aedes polynesiensis*, and several species of the *Aedes scutellaris* complex. These secondary vector species can transmit outbreaks, but are more important as maintenance vectors. Dengue viruses are also maintained in forest cycles in Asia and Africa. The secondary vector species has its own particular geographical distribution; however, they are less efficient epidemic vectors than *Aedes aegypti.* While vertical (transovarian) transmission of dengue viruses has been demonstrated in both the laboratory and the field. A factor complicating eradication of the vector is that *Aedes aegypti* eggs can withstand long periods of desiccation, sometimes for more than a year (5).

3. Transmission of dengue virus

Dengue viruses are transmitted to human through the bite of infected *Aedes* mosquitoes, principally *Aedes aegypti.* The adult mosquitoes prefer to rest indoors and prefer to feed on humans during daylight hours. There are two peaks of biting activity, early morning for 2 to 3 hours after daybreak and in the afternoon for several hours before dark. However, these mosquitoes will feed all day indoor and overcast days (8, 49). In infection mosquitoes, dengue viruses multiply in the midgut epithelium, brain, fat body, and salivary glands. No detectable pathologic changes result from infection, and mosquitoes remain infectious for life. Dengue virus replicates in the female mosquito

genital tract and may enter the ovum at the time of fertilization, thereby infecting a portion of her progeny (7). Infected female mosquitoes may also pass the virus to the next generation of mosquitoes by transovarian transmission, but this occurs infrequently and probably does not contribute significantly to human transmission. Sexual transmission may be occurs from male *Aedes* with inherited infections to susceptible females, which may subsequently pass the virus to their progeny. The female mosquitoes are very nervous feeders, disrupting the feeding process at the slightest movement, only to return to the same or a different person to continue feeding moments later. Because of this behavior, *Aedes aegypti* females will often feed on several persons during a single blood meal and, if infective, may transmit dengue virus to multiple persons in a short time, even if they only probe without taking blood. It is behavior that makes *Aedes aegypti* such an efficient epidemic vector. Humans are the main amplifying host of the virus, although studies have shown that monkeys in some parts of the world may become infected and perhaps serve as a source of virus for feeding mosquitoes. After an infective mosquito bites person, the virus undergoes an incubation period of 3 to 14 days (average, 4 to 7 days), after which the person may experience acute onset of fever accompanied by a variety of non-specific signs and symptoms. The virus circulates in the blood of infected humans at approximately the time that they have fever, and uninfected mosquitoes may acquire the virus if they feed on an individual when he or she is viremic. Those mosquitoes may become infected and subsequently transmit the virus to other uninfected persons, after an extrinsic incubation period of 8 to 12 days (5, 8, 49).

4. Epidemiology

Dengue viruses occur worldwide in tropical regions; their distribution is determined by the presence of the principal mosquito vector. In tropical areas, the vector is active year-round and dengue occurs throughout the year, with increased transmission

during the rainy season. This is due to higher mean temperatures and attendant shorter extrinsic incubation period in the vector (interval between feeding on infectious blood and ability to transmit on refeeding) and to higher humidity and enhanced survival of adult mosquitoes. Temperate areas within the range of *Aedes aegypti* are susceptible to summertime introduction and spread of the virus. In areas having year-round vector activity and large human populations, one or more dengue virus types may be maintains endemically. Vector density and factors determining exposure to infected female mosquito vectors are important determinants of rate of dengue virus transmission.

Epidemic of an illness compatible with dengue fever was first reported in the medical literature in 1779 and 1780. During the Second World War Southeast Asia experienced the co-circulation of multiple dengue virus serotypes and endemic activity increased. The disease was first recognized in the Philippines in 1953. The syndrome was etiologically related to dengue viruses when serotypes 2, 3, and 4 were isolated from patients in the Philippines in 1956; 2 years later dengue viruses of multiple types were isolated from patients during an epidemic in Bangkok, Thailand. During the next three decades, DHF/DSS was recognized in Cambodia, China, India, Indonesia, The Lao People's Democratic Republic, Malaysia, Maldives, Myanmar, Singapore, Sri Lanka, Vietnam, and several Pacific Island groups. With subsequent uncontrolled growth of cities, epidemic DHF emerged as a major public health problem in most countries of Southeast Asia and Pacific Island groups. During 1960s and 1970s, DHF/DSS progressively increased spreading from its primary location in major cities to smaller cities and towns in endemic countries. It established seasonal and cyclical epidemic patterns, with large outbreaks occurring at 2-3 year intervals. During this period, 1070207 cases and 42808 deaths were reported mostly in children. During most of the 1980s, in the endemic countries of China, Indonesia, Malaysia, Myanmar, Philippines, Thailand, and Vietnam, DHF/DSS spread principally, affecting even rural villages. Exceptionally

large outbreaks occurred in Vietnam (354577 cases in 1987) and Thailand (174285 cases in 1987). In many countries, DF and DHF are primarily diseases of children, since they represent the largest segment of susceptible individuals within the population at risk. Increasingly, DF, and occasionally DHF, is also seen among travelers. The disease is among the ten leading causes of hospitalization and death in children in at least eight tropical Asian country (5). Intermittent transmission of dengue has occurred in Queensland, Australia, outbreaks of dengue-2 occurred in the Seychelles Island, Coastal Kenya, Saudi Arabia, and Yemen. These epidemics were spawned originally by virus introduction from Asia, but the virus is now endemic in the Horn of Africa. The worldwide incidence of dengue has increased dramatically in the period following World War II, due to expanding urban human populations and a coincident increase in *Aedes aegypti* density, as well as the advent of air travel and rapid movement of viremic persons. These changes are underscored in the Americas, where the frequency of epidemics has increased dramatically and multiple dengue serotypes have been introduced. In 1981 an outbreak of DHF/DSS occurred in Cuba that marked the start of DHF in the region on the Americas. In the past, the continent of South America was reinvaded by *Aedes aegypti*, with the result that large dengue outbreak, occurred in immunologically naïve populations. In hyperendemic areas of Southeast Asia, over 50%of children experience infection with one or more dengue serotypes by age 7. In these areas, dengue is a childhood disease, and adults are protected by cumulative immunity.

In Thailand, outbreaks first occurred in Bangkok in a pattern with a 2-year cycle then subsequently in irregular cycle as the disease spread throughout the country. DHF then became endemic in many large cities of Thailand, eventually spreading to smaller towns and villages during periods of epidemic transmission. Two important epidemiological patterns have been recognized. First, DHF/DSS has appeared most frequently in areas where multiple dengue serotypes are endemic. The usual pattern is that

of sporadic cases or small outbreaks in urban areas that steadily increase in size until there is an explosive outbreak that brings the disease to attention of public health authorities. The disease then usually establishes a pattern of epidemic activity every 2-5 years. In addition, DHF/DSS is typically confined to children, with a modal age at hospitalization of 4-6 years. A second pattern is observed in areas of low endemicity. Multiple dengue serotypes may be transmitted at relatively low rates of infection (below 5% of the population per year). In these areas, previously uninfected adults are susceptible to dengue infection, and children and young adults, with a modal age of 6-8 years, are also vulnerable. A cyclical pattern of increased transmission coinciding with the rainy season has been observed in some countries. The interactions between temperature and rainfall are important determinants of dengue transmission, as cooler temperatures affect adult mosquito survival, thus influencing transmission rates. Furthermore, rainfall and temperature may affect patters of mosquito feeding and reproduction, and hence the population density of vector mosquitoes (2, 5, 6, 9, 16, 50, 51, 52, 53, 54, 55, 56, 57, 58).

5. Pathogenesis

The pathogenesis of dengue infection is still controversial. Three hypotheses about these, which are not mutually exclusive, are frequently cited to explain the pathogenesis changes that occur in DHF and DSS.

1) Immune enhancement

The regulation of severity of dengue disease is that DF and DHF/DSS syndromes differ in the number of cells infected; the greater the number of infected cells, the more severe the disease. Heterotypic antibody circulating in blood at the onset of dengue infection forms infectious virus-antibody complexes and enhance the efficiency of entry of viruses into cells of mononuclear phagocyte lineage, the putative target cell system (59).

The immune response to dengue infection plays an important role in determining the outcome and severity of disease. The viremic period is terminated coincident with the appearance of serum antibodies. In most patients, humoral and cellular responses result in recovery from infection and long-lasting protection against reinfection with the homologous serotype. Serotype-specific neutralizing antibodies directed against the E protein and serotype-specific CD4+ and CD8+ memory cytotoxic lymphocytes directed against structural and nonstructural viral targets on infected cells are responsible for protection and recovery. Cross-protection against other dengue serotypes is short-lived, and in a subset of individuals experiencing secondary infection with a heterologous serotype, a severe immunopathologic disease (dengue hemorrhagic fever) occurs and is mediated by the interaction of antibodies and T cells.

The most commonly accepted is known as the secondary-infectious or immune enhancement hypothesis. This hypothesis developed extensively by Halstead (10) implies that patients experiencing a second infection with a heterologous dengue virus serotype have a significantly higher risk for developing DHF and DSS. Preexisting heterologous dengue antibody recognizes the infecting virus and forms an antigen-antibody complex, which is then bound to and internalized by immunoglobulin Fc receptors on the cell membrane of leukocytes, especially macrophages. Because the antibody is heterologous, through, the virus is not neutralized and is free to replicate inside the macrophage (54, 55, 60). Thus, it is hypothesized that prior infection, through a process known as antibody-dependent enhancement (ADE), enhances the infection and replication of dengue virus in cells of the mononuclear cell lineage. It is thought that these cells produce and secret vasoactive mediators in response to dengue infection, which cause increased vascular permeability leading to hypovolemia and shocks (4, 10, 11, 14, 61).

2) Specific viral serotype

In recent years, a more prominent role of viral factors in the pathogenesis of DHF are suggesting that virus strain and serotype are also important risk factors for severe disease. Dengue hemorrhagic fever or dengue shock syndrome only occurs in a relatively small proportion of patients with secondary infections. Despite the co-circulation of several dengue serotypes in the Americas, it was not until the 1981 epidemic in Cuba that the first DHF cases occurred in the region. This event coincided with the introduction of a new genotype of dengue virus serotype 2 from Southeast Asia. Subsequent epidemics with DHF in South America also coincided with the occurrence of South Asian dengue virus strains. In contrast, in Peru, no evidence of DHF was found during an epidemic caused by dengue 2 virus, five years after an epidemic of dengue 1. Evidence of secondary dengue virus infection was found in 60.5% of subject tested. The absence of DHF in this population has been attributed to the American origin of the dengue 2 strain causing the epidemic. These and other findings suggest that viral virulence factors may play an essential role in the pathogenesis of DHF/DSS (13, 61).

3) Nutritional status

The previous study by Usa T. and Suchitra N. showed that there are relationship between nutritional status and clinical manifestation of dengue infection. They had suggested that patients with DHF had better nutritional status when compared with other infection disease and healthy children. Besides, they confirmed that patients with DHF are not usually malnourished (62).

6. Clinical manifestation

Dengue infection characteristically results in fever, headache, and rash. The clinical spectrum can vary, however, from asymptomatic to more severe infections with bleeding and shock. They can be classified into four presentations: non-specific febrile illness, classical dengue, dengue hemorrhagic fever, and dengue hemorrhagic fever with dengue shock syndrome. Asymptomatic and classical dengue is more commonly seen among older children, adolescents, and adults. Dengue is abrupt in onset, typically with high fever accompanied by severe headache, incapacitating myalgias and arthralgias, nausea and vomiting, and rash. A positive tourniquet test may be found in over one-third of patients with DF. Clinical findings alone are not very helpful to distinguish DF from other febrile illness such as chikungunya and measles. Infection of any of the four serotypes causes a similar clinical presentation that may vary in severity, depending on a number of risk factors. The incubation period varies from 3 to 14 days (average, 4 to 7 days). In areas where is endemic, the illness is often clinically nonspecific, especially in children, with symptoms of a viral syndrome that has a variety of local names. Important risk factors influencing the proportion of patients who have severe disease during epidemic transmission include the strain and serotype of the infecting virus and the immune status, age, and genetic background of the human host.

Classical dengue fever is primarily a disease of older children and adults. It is characterized by the sudden onset of fever and a variety if nonspecific signs and symptoms, including frontal headache, retro-orbital pain, body aches, nausea and vomiting, joint pains, weakness, and rash. Patients may be anorexic, have altered taste sensation, and have a mild sore throat. Hemorrhagic manifestations in dengue fever patients are not uncommon and range from mild to severe. Skin hemorrhages, including petechiae and purpura, are the most common, along with gum bleeding, epistaxis, menorrhagia, and gastrointestinal hemorrhage. Clinical laboratory findings associated

with dengue fever include a neutropenia followed by a lymphocytosis, often marked by elevated; the elevation is usually mild. Thrombocytopenia is also common is dengue fever. Dengue fever is generally self-limiting and is rarely fatal. The acute phase of illness lasts for 3 to 7 days, but the convalescent phase may be prolonged for weeks and may be associated with weakness and depression, especially in adults. No permanent sequelae are known to be associated with this infection.

Dengue hemorrhagic fever is primarily a disease of children under 15 years in hyperendemic areas. DHF is defined as an acute febrile illness with minor or major bleeding, thrombocytopenia ($\leq 10^5/\mu l$), and evidence of plasma leakage documented by hemoconcentration, pleural or other effusions, or hyperalbuminemia, or hypoproteinemia. The major pathophysiological change that determines the severity of disease in DHF and differentiates it from DF is the leakage from plasma. Transudation due to excessive capillary permeability collects at the pleural and abdominal cavities. The development if DHF provides warning of an increased probability of shock. The first to ascertain is the time elapsed since onset of illness. DHF/DSS usually develop around day 3-7 of illness, at the time of defervescence, which is an indication for intensified observation. A progressively decreasing platelet count and rising hematocrit indicate increased probability of impending shock. When all four criteria for DHF are fulfilled, intravenous fluids may be all that is requires for treatment. Dengue shock syndrome is defined as DHF with signs of circulatory failure, including narrow pulse pressure ($\leq$ 20mmHg), hypotension or frank shock. The four warning signs for impending shock are intense, sustained abdominal pain; persistent vomiting; restlessness or lethargy; and a sudden change from fever to hypothermia with sweating and prostration. Early diagnosis and aggressive fluid replacement therapy with good nursing care can decrease fatality rate (1, 5, 13, 14, 47, 59, 63, 64, 65).

7. Laboratory diagnosis (5)

A definitive diagnosis of dengue infection depends on isolating the virus, viral antigen detection, viral nucleic acid detection, and specific antibody detection from patient's specimen, often be serum. Acute-phase blood sample is a choice for diagnosis of dengue infection. So, blood sample should be taken in during onset of suspected dengue illness and ideally, should be taken for convalescent-phase sample 2-3 weeks later, however, it is often difficult, but should be taken blood sample before discharged from hospital.

1) Serological diagnosis

Routinely serologic tests for dengue infection's diagnosis are hemagglutination-inhibition (HI), immunoglobulin M (IgM) capture enzyme-linked immunosorbant assay (MAC-ELISA), indirect immunoglobulin G ELISA, neutralization test (NT), and complement fixation test (CF).

Hemagglutination-inhibition assay (HI) has been the most frequently used; it is sensitive, is easy to perform, requires only minimal equipment, and is very reliable if properly done. Because HI antibodies persist for long periods, the test is ideal for seroepidemiologic studies. HI antibody usually begins to appear at detectable levels, about titer of 10, after 5-6 days of illness. In primary infection, antibody titers in convalescent-phase serum often are less than 640. But in secondary infection, reciprocal antibody titer increase rapidly during the first few days of illness by immediate anamnestic response, often reached to more than 5,210. Thus, a titer of $\geq$ 1,280 in an acute-phase or early convalescent-phase serum sample is considered presumptive evidence of a current dengue infection. The disadvantage of HI test is lack of specificity, unreliable for identifying the infecting virus serotype.

An indirect IgG-ELISA, this assay is comparable to HI test and can also be used to differentiate primary and secondary dengue infection. The test is simple and easy to perform for high-number testing. It's very nonspecific and exhibits the same broad cross-reactivity among *Flavivirus* as the HI test does. Therefore, it can not be used to identify the infecting dengue virus serotype. However, it has a slightly higher sensitivity than HI test. As more data are accumulated on the IgG-ELISA, it is expected to replace the HI test as the most commonly used IgG test in dengue laboratories (66).

The complement fixation (CF) test is not widely used for routine dengue diagnostic serological testing. It is more difficult to perform, requires highly trained personnel, and therefore is not used in most dengue laboratories. It is based on the principle that complement is consumed during antigen-antibody reaction. CF antibodies generally appear later HI antibodies, are more specific in primary infections, and usually persist for short periods, although low level of antibodies persists in some persons. The CF test is useful for patients with current infections but is of limited value for seroepidemiologic studies, where detection of persistent antibodies is important.

The neutralization test is the most specific and sensitive serologic test for dengue viruses. The most common protocol used in dengue laboratories is the serum dilution plaque reduction NT. In general, neutralizing-antibody titers rise at about the same time or slightly more slowly than HI and ELISA antibody titers but more quickly than CF antibody titers and persist for at least 48 years. Because the NT is more sensitive, neutralizing antibodies are present in the absence of detectable HI antibodies in some persons with past dengue infection. The NT can be used to identify the infecting virus in primary dengue infection. The major disadvantages are the expense, time required to perform the test, and technical difficulty.

MAC-ELISA has become the most widely used serologic test for dengue diagnosis. It is a simple, rapid test that requires very little sophisticated equipment. Anti-dengue IgM antibody develops a little faster than IgG antibody. By 5 days of illness, HI subsequently confirmed most patients on pair serum samples or by virus isolation had detectable IgM antibody in the acute phase serum in this assay. Nearly all patients (93%) developed detectable IgM antibody 6 to 10 days after onset, and 99% of patients tested between 10 and 20 days had detectable IgM antibody. The specificity of MAC-ELISA is similar to that HI. In general, the response is broadly reactive among both dengue virus and other flavivirus antigen. With serum samples from patients with other flavivirus infections such as Japanese encephalitis, St. Louis encephalitis, and yellow fever, however, the response is generally more specific; while there may be some cross-reaction with dengue antigens, most specimens show relatively monotypic IgM response to the infecting flavivirus. In dengue infections, monotypic IgM responses frequently do not correlate with the virus serotype isolated from a patient. Therefore, MAC-ELISA cannot be reliably used to identify the infecting virus serotype.

2) Viral isolation

By period of viremic, isolation of dengue virus from clinical specimens is frequently possible. Factors favoring the successful isolation of virus are collections of the specimen early in the course of disease (usually within 5 days after the onset of fever) and proper handling a prompt delivery of specimen to the laboratory. There are different methods (Table 2) of confirming the dengue virus, choice of method up to local availability of mosquito, cell culture and mice.

Newborn mice, intracerebral inoculation of 1 to 3 day old baby mice, although all four dengue serotypes were initially isolated from human serum by using baby mice, this method is very time-consuming, slow, and expensive. Moreover, because of the low

sensitivity of the method, many wild type viruses cannot be isolated with baby mice. Those that are isolated frequently require numerous passages to adapt the viruses to growth in mice. This method is no longer recommended for isolation of dengue viruses, but some laboratories continue to use it. One advantage of using baby mice, however, is that other arboviruses that cause dengue-like illness may be isolated with this system.

Table 2 Methods for dengue virus isolation

Method	Result confirming presence of dengue virus
Inoculation of mosquito (Adult or larvae)	Detection of antigen in head squash by serotype-specific immunofluorescence
Inoculation of various Mammalian or insect Cell culture	Detection of antigen by antibody staining Cytopathic effect; identification of virus upon subpassage Plaque formation; identification of virus upon subpassage
Intracranial inoculation of Sucking mice	Presence of antigen in brain detected by antibody staining Symptoms or signs indicating encephalitis Identification of virus upon subpassage

Mammalian cell cultures have many of the same disadvantages as baby mice for isolation of dengue viruses; they are expensive, slow, and insensitive. As with isolation systems that use baby mice, viruses that are isolated frequently require many passages before a consistent cytopathic effect can be observed in the infected cultures. Although the use of this method continues in some laboratories, it is not recommended.

Mosquito inoculation is the most sensitive method for dengue virus isolation. Isolation rates of up to 100% of serologically confirmed dengue infections are not uncommon, and this is the only method sensitive enough for routine successful virologic

conformation of fatal DHF and DSS cases. Moreover, there are many endemic dengue virus strains that can be recovered only by this method. Four mosquito species have been used for virus isolation, *Aedes aegypti, Aedes albopictus, Toxorhynchities amboinensis,* and *Toxorhynchities splendens*. Male and female mosquitoes are equally susceptible; dengue viruses generally replication to high titer in as little as 4 to 5 days. Dengue virus replicate in most mosquito tissue, including the brain. Virus detection in the mosquito, regardless of the species, is generally performed by the direct fluorescent-antibody (DFA) test on mosquito tissue, usually brain or salivary glands. The mosquito inoculation technique has the disadvantages of being labor-intensive and requiring an insectary to produce large numbers of mosquitoes for inoculation. Also, unless strict safety precautions are maintained, the chance of laboratory infections increases, although this risk can be eliminated by using male *Aedes* mosquitoes or nonbiting *Toxorhynchities* species for inoculation.

Mosquito cell culture are the most recent additional to dengue virus isolation methodology. Three cell lines of comparable sensitivity are most frequently used. The first cell line developed, and still the most widely used, is the C6/36 clone *of Aedes albopictus* cells. The use of these cell lines has provided a rapid, sensitive, and economical method for dengue virus isolation. Moreover, many serum specimens can be processed easily, making the method ideal for routine virologic surveillance. However, this system is less sensitive than mosquito inoculation. However, the sensitivity of the mosquito cell lines may vary with the strain of virus. DFA or IFA tests can detect dengue antigen in infected-cell cultures with the conjugates used for mosquito tissue. Some workers, however, prefer to use cytopathic effect to detect infection. However, this method alone will miss many dengue viruses that so not replicate rapidly in mosquito cells.

3) Viral antigen detection

This is a new method of immunohistochemistry, it is now possible to detect dengue viral antigen in a variety of tissues. Although immunofluorescence tests were used in the past, newer methods involving enzyme conjugates such as peroxidase and phosphatase in conjugation with either polyclonal or monoclonal antibodies are greatly improved. Because tissue can be fresh or fixed, autopsies should be performed in all cases of suspected DHF with a fatal outcome.

4) Viral nucleic acid detection (67, 68, 69)

In recent year, several new method of diagnosis has been developed and has proven very useful in diagnosis, such as Polymerase Chain Reaction (PCR) and hybridization probe.

PCR. Reverse transcriptase polymerase chain reaction (RT-PCR) has been developed for a number of RNA viruses in recent years and has the potential to revolutionize laboratory diagnosis; for dengue, RT-PCR provides a rapid serotype-specific diagnosis. The assay is rapid, sensitive, simple, and reproducible if properly controlled and can be used to detect viral RNA in human clinical samples, autopsy tissue, or mosquitoes. Although RT-PCR has similar sensitivity to virus isolation systems that use C6/36 cell cultures, poor handling, poor storage, and the presence of antibody usually do not influence the outcome of PCR as they do virus isolation. A number if methods involving primers from different approaches to detect the RT-PCR products have been developed over the past several years.

Hybridization Probes. The hybridization probe method detects viral nucleic acids with cloned hybridization probes. Probes with variable specificity ranging from dengue complex to genotype specific can be constructed depending on the genome sequences used. This method is rapid and relatively simple and can be used on human

clinical samples as well as fixed autopsy tissues. Preliminary data suggest that this method is less sensitive than RT-PCR.

8. Vaccine development

The World Health Organization designated the development of a tetravalent dengue vaccine a priority for the most cost-effective approach to dengue prevention. Effective vaccination to prevent DHF will most probably require a tetravalent vaccine, because epidemiologic studies have shown that preexisting heterotypic dengue antibody is a risk factor for DHF. With WHO supported, developing dengue vaccine has been made in recent years. Promising candidate attenuated vaccine viruses have been developed and has been evaluated in phase I and II trials in Thailand as monovalent, bivalent, trivalent, and tetravalent formulations (70). A commercialization contract has been signed, and the tetravalent vaccine formulation is currently undergoing repeat phase I trials in the United States. Current progress on the live attenuated dengue vaccine has been recently reviewed.

In recent years, molecular technology was used to modify vaccine strategies. Additional, inactivated whole virion vaccine, synthetic peptides, subunit vaccine, vector expression, recombinant live vector systems, infectious cDNA clone-derived vaccine and naked DNA were developed in vaccine strategies. The last two approached appear to be the most promising. An infectious clone of the DEN-2, PDK-53 vaccine candidate virus from Thailand has been constructed, and work is in progress to construct chimeric viruses by inserting the capsid, pre-membrane, and envelope genes of DEN-1, DEN-3, and DEN-4, into the DEN-2 PDK-53 backbone. Despite the promising progress, it is unlikely that an effective, safe, and economical dengue vaccine will be available in the near future (5).

II. LightCycler System (23)

The LightCycler is an extremely fast thermocycler with online fluorescence detection. It enables the user to monitor the amplification of the PCR product simultaneously in real-time allowing the accurate quantification of target sequences. The innovation function called melting curve analysis performed alter the PCR offers a powerful system for genotyping, single nucleotide polymorphisms (SNP), and for mutation detection. The LightCycler consists of a cylindrical chamber where the air, which is warmed by a heating coil. The PCR occurs in glass capillaries, which can hold up to a volume of 20 µl. This high surface to volume ratio allows very rapid cycles. The detection unit consists of a blue light emitting diode (LED: 470 nm) light source and three detection channels which measure emitted light at three different wave lengths (F1 = 530 nm, F2 = 640 nm, and F3 = 710 nm). The ability of the LightCycler is to perform a detailed melting curve analysis of the PCR products subsequent to amplification run. The melting point of a DNA fragment depends on its individual length and its G/C content. The determination of the individual melting temperature for a DNA fragment can be used to characterize the amplification products. The system, which comes with data analysis software, uses detection of fluorescence to monitor product amplification. It is set up for 3 formats, using the double stranded DNA (dsDNA) binding dye SYBR Green I, hybridization probes, and hydrolysis probe.

SYBR Green I is dsDNA binding dye. It is thought to bind in the minor groove of the dsDNA and upon binding increases in fluorescence over a hundred folds. In the LightCycler SYBR is monitored in channel F1. Since SYBR Green I dye is very stable and the LightCycler instrument's optical filter set matches the wavelengths of excitation and emission, it is the reagent of choice when measuring total DNA. The principle is outlined in the following. At the beginning of amplification, the reaction mixture contains the denatured DNA, the primers, and the dye. The unbound dye molecules weakly

fluorescence, producing a minimal background fluorescence signal, which is subtracted during computer analysis. After annealing of the primers, a few dye molecules can bind to the double strand. DNA binding results in a dramatic increase of the SYBR Green I molecules to emit light upon excitation. During elongation, more dye molecules bind to the newly synthesized DNA. If the reaction is monitored continuously, an increase in fluorescence is viewed in real-time. Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls. Fluorescence measurement at the end of the elongation step of every PCR cycle is performed to monitor the increasing amount of amplified DNA. The disadvantage of SYBR Green I is that it binds to any dsDNA; the specific product, nonspecific products and primer-dimers are detected equally well. Actually, the LightCycler allows melting curve analysis of the reaction. This can help to determine the fraction of the signal coming from the desired product and the fraction coming from primer-dimer. Once the melting point of the product has been determined the LightCycler's flexible programming allows the user to acquire fluorescence above the melting temperature of the primer-dimers, but below the melting temperature of the product (25, 27, 28, 31, 32, 34, 36, 71,72).

Hydrolysis probes or TaqMan probes are singled oligonucleotides that can bind to the amplicon and are modified to contain a fluorophore are quencher 3 to 30 bases apart. During the amplification the probe will be hydrolyzed by a double strand specific nuclease activity associated with the *Taq* polymerase, 5' to 3' exonuclease activity, resulting in a dissociation of reporter from quencher - the fluorescence increase. These probes can be used in the LightCycler and are monitored in F1 or F1/F2 (39).

Hybridization probes are used for DNA detection and quantification and provide a maximal specificity for product identification. In addition to the reaction components used for conventional PCR, two specially designed, sequence specific oligonucleotides labeled with fluorescent dyes are applied for this detection method. This allows highly

specific detection of the amplification product. The three essential components for using fluorescence-labeled oligonucleotides: two oligonucleotides and the amplification product. The oligonucleotide 1 carries a fluoresein label at its 3'end whereas oligonucleotide 2 carries another label (LC-Red 640) at its 5'end. The sequences of the two oligonucleotides are selected such that they hybridize to the amplified DNA fragment in a head to tail arrangement. When the oligonucleotides hybridize in this orientation, the two fluorescence dyes are positioned in close proximity to each other. The LightCycler excites the first dye (fluoresein); LED filtered light source, and emits green fluorescent light at a slightly longer wavelength. When the two dyes are in close proximity, the emitted energy excites the LC-Red 640 attached to second hybridization probe that subsequently emits red fluorescent light at an even longer wavelength. Thus energy transfer referred to, as Fluorescence Resonance Energy Transfer (FRET) is highly dependent on the spacing between the two dye molecules. Only if the molecules are in close proximity (a distance between 1-5 nucleotides) is the energy transferred at high efficiency. The intensity of the light emitted by the LightCycler-Red 640 is filtered and measured by the LightCycler instrument's optics. The increasing amount of measured fluorescence is proportional to the increasing amount of DNA generated during the ongoing PCR process. Since LC-Red 640 only emits a signal when both oligonucleotides are hybridized, the fluorescence measurement is performed after the annealing step. Hybridization probes can be labeled with LightCycler-Red 640 and with LightCycler-Red 705. In the LightCycler LC-Red 640 are monitored in channel F2, LC-Red 705 in channel F3 (26, 38, 40, 41, 42, 43, 44, 45, 46, 73).