CHAPTER II

LITERATURE REVIEW

Dengue Structure and nonstructural proteins.

Dengue viruses have four distinct serotypes designated DEN-1, DEN-2, DEN-3, and DEN-4 which can be distinguished by serological and molecular method. The particle are spherical, lipid-enveloped viruses that contain a positive sense single-strand RNA genome of approximately 10,200 nucleotides (~11 kb) in length composed of three structural protein genes encoding the nucleocapsid or core protein (C), a membrane associated protein (M), an envelope protein (E), and seven non-structural (NS) protein, the complete genome sequence was know for isolates of four dengue virus serotypes (2, 6, 9, 24, 25, 26). The order of proteins is 5'-C-prM (M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3' (figure 1) (27). The viral proteins have many function such as capsid is a viral RNA (vRNA) packing, pre-membrane is prevention of premature fusion, envelope is receptor binding or fusion, NS1 is signal transduction, NS2B is NS3 serine protease cofactor, NS3 is helicase; NTPase; 5' triphosphatase; serine protease, NS4B is inhibition of interferon (IFN) signal transduction, and NS5 is RNA polymerase; methyltransferase (5).

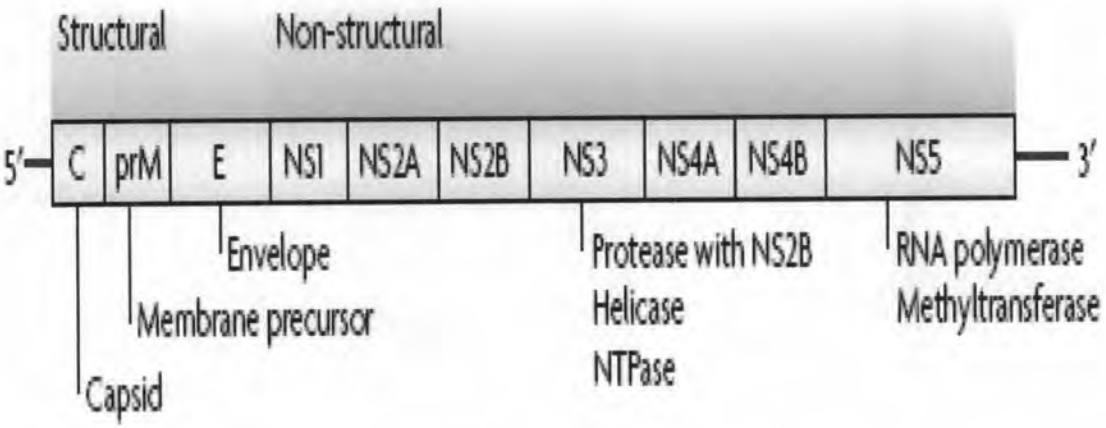


Figure 1. Structure of the dengue virus genome. Dengue vRNA contains a 5' type 1 cap structure, and the single open reading frame is flanked by 5' and 3' UTRs. Both 5' and 3' untranslated region (UTR) have key roles in the regulation of translation and genomic RNA synthesis (32)

Dengue virus replication

Infection with the one of dengue virus begins when the vector takes the blood meal and the virus is introduced into the host. The virus binds to and enters a permissive host cell via receptor-mediated endocytosis. Upon internalization and acidification of the endosome fusion of viral and vesicular membranes allos entry of the nucleocapsid into the cytoplasm and genome uncoating. Translation of the input strand takes place then the virus switches from translation to synthesis of the negative-strand intermediate which serves as a template for the production of the multiple copies of positive-strand viral RNA (vRNA). Successive rounds of translation produce high levels of proteins the structural protein capsid or core (C), premembrane (prM), and envelope (E) proteins along with vRNA are assembled into progeny virions which are transported through the Golgi compartment and secreted (figure 2) (5). It is believed that the cells that mainly support dengue virus infections *in vivo* are monocytes, macrophages and other cell of reticuloendothelial origin (28, 29). It has been reported recently that dendritic cells, manely Langerhans cells, are more permissive for dengue virus than monocytes and macrophages (30, 31). Although there were reports that fibroblasts, hepatocytes and B cells were infected with dengue viruses (32, 33, 34), it is likely that

dendritic cells and monocytes/macrophages are the cell that mainly support dengue virus infection *in vivo*. Vascular endothelial cells can be infected with dengue virus in vitro. The main target organs of dengue virus infections are not known. The liver appears to play a role in dengue virus propagation *in vivo*; however, whether the liver is the main organ of dengue virus propagation has not been defined yet.

Figure 2. Intracellular life cycle of dengue virus. DENV bind (step1) and enters (step 2) cell via an uncharacterized receptor by receptor mediated endocytosis (RME). Endosomal acidification (step 3) results in an irreversible trimerization of the viral E protein, exposing the fusion domain. After being uncoated, the positive viral strand RNA (vRNA) is translated (step 4) at ER-derived membranes, where it is processed into three structural and seven non-structural proteins. After the viral replication complex is synthesized, vRNA translation switches off and RNA synthesis (step 5) begins. Subsequently, successive rounds of translation (step 6) are followed by assembly in the ER. The virion is maturated in the Golgi compartment (step 7) and exits via the host secretory pathway (5).

Dengue Epidemiology

After 1945, dengue cases complicated by hemorrhage and shock (DHF and DSS) were increasingly documented in Southeast Asia. The first report DHF epidemic occurred in the Philippine Islands in 1953-1954, after that epidemic activity of DHF intensified in that region, although it remained confined to Southeast Asia through the 1970s, however, epidemic DHF spread west into India, Pakistan, Sri Lanka, and the Maldives and east into China (35, 36).

In the Americans, dengue was not considered a major public health problem until recent years. Attempts to eradicate A. *aegypti* from the Americas were undertaken in the 1950s. Although this program, coordinated by the Pan American Health Organization, was successful in several countries, it failed to eradicate A. *aegypti* from the whole region. After support for mosquito surveillance and control programs were reduced, most countries became re-infested with A. *aegypti* by the end of the 1970s. This, along with the emergence of new dengue serotypes, resulted in major epidemics throughout the region and the emergence of DHF. In 1981, the first major DHF epidemic in the Americas occurred in Cuba. During that outbreak, an estimated 344,000 dengue infections occurred and over 116,000 hospitalized patients and 10,000 cases of DHF were reported from May to October (37). Since the re-infestation of Central and South America, epidemics caused by multiple serotypes (hyperendemicity) were more frequent and the geographic distribution of dengue viruses and their mosquito vectors had expanded. Moreover, by 1997, DHF had emerged as a disease entity in several major and many minor epidemics in tropical and subtropical countries of the Americas (Figure. 3). Today an estimated 50-100 million cases of dengue fever and 500,000 cases of DHF, resulting in around 24,000 deaths, occur annually, depending on the epidemic activity (38, 39). Over half the world's population lives in areas potentially at risk for dengue transmission, making dengue the most important human viral disease transmitted by arthropod vectors in terms of morbidity and mortality.

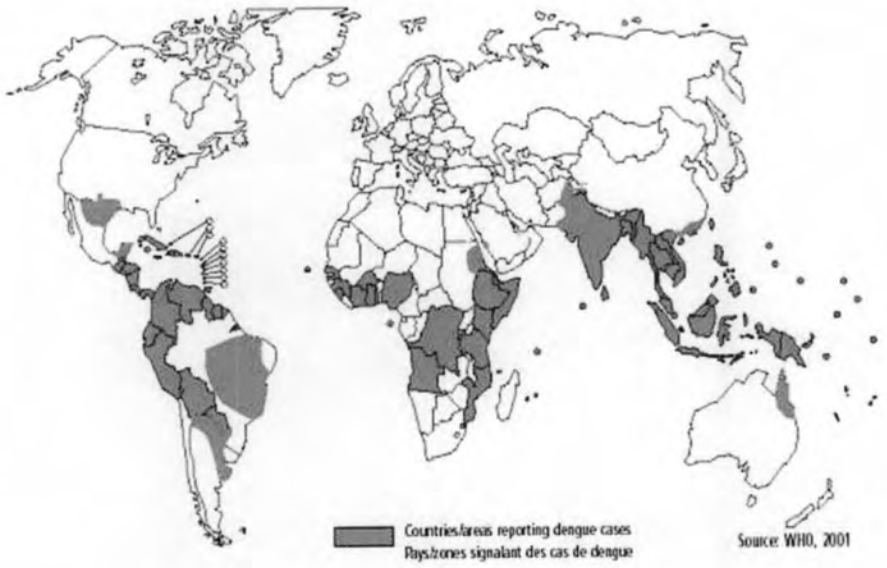


Figure3. Countries/areas reporting dengue cases of the World Health Organization in 2001.

The definition and severity classification of disease

The WHO scheme classifies was formulated by Eva Harris and Jacqueline Deen in 2006 for classifies symptomatic dengue virus infection into three categories as undifferentiated fever, dengue fever, and DHF (figure 4.) (40).

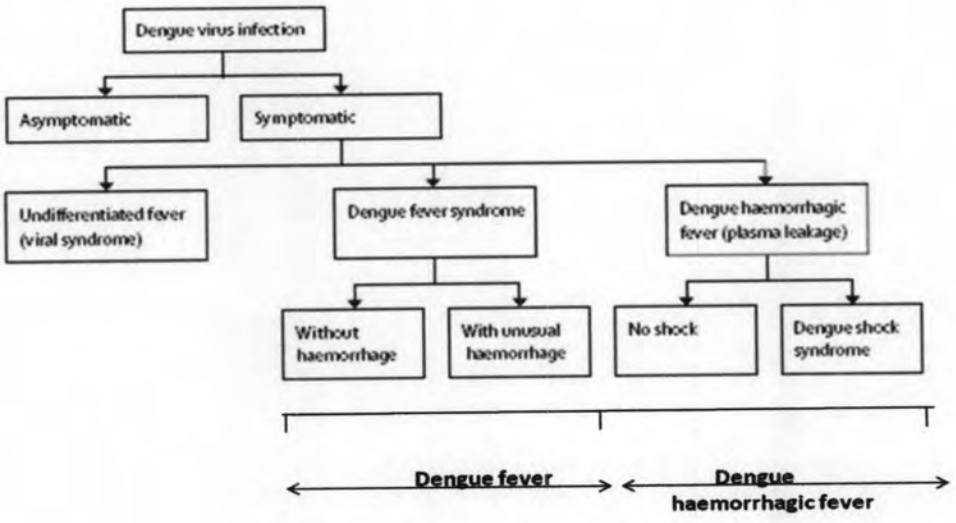


Figure 4. Who classification of symptomatic dengue infection (40)

Dengue fever is clinically defined as an acute febrile illness with two or more manifestations (headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, or leucopenia) and occurrences at the same location and time as other confirmed cases of dengue fever. A case must meet all four of the following criteria to be define as DHF (table 1) Present fever or history of fever lasting 2-7 days; a hemorrhagic tendency shown by a positive tourniquet test or spontaneous bleeding (petichiae, ecchymosis, purpura, hematemesis, melena), thrombocytopenia (platelet count $100+10^9$ /L or less), and evidence of plasma leakage shown either by haemoconcentrations of packed-cell volume, or by the development of pleural effusions or ascites, or both. Severity grading of DHF is defined into 4 grades (Figure.5)

Grade I. Fever accompanied by 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 manifestations of Grade I patients, usually in the forms of skin or other hemorrhages.

Grade III. Circulatory failure manifested by a rapid, weak pulse and narrowing of pulse pressure or hypotension, with the presence of cold, clammy skin and restlessness.

Grade IV. Profound shock with undetectable blood pressure or pulse

The term dengue shock syndrome (DSS) refers to DHF grades III and IV in which shock is present as well as all four DHF definition criteria. Moderate shock identified by narrowing of the pulse pressure or hypotension for age, is present in grade III DHF, whereas profound shock with no detectable pulse or blood pressure is present in grade IV DHF. The incubation period before the development of sign of infection generally ranges from 4 to 7 days. Hypovolemic shock can develop during the late stage of disease and usually lasts 1 to 2 days (Table.1) (27).

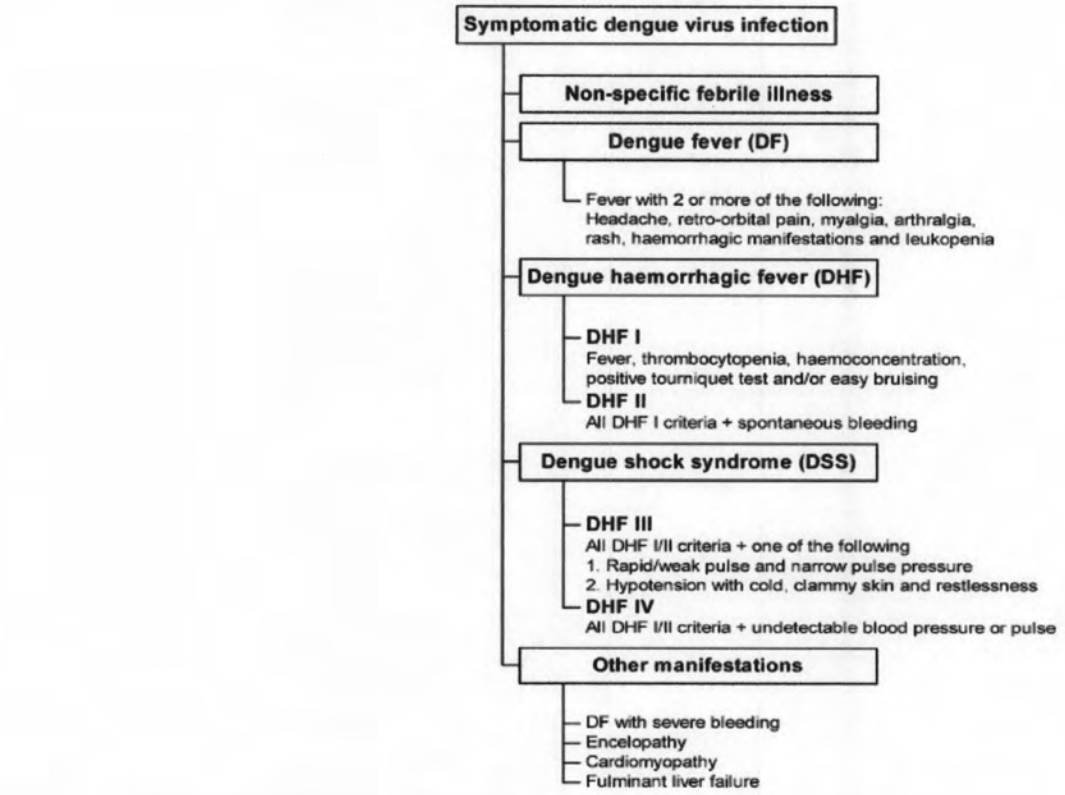
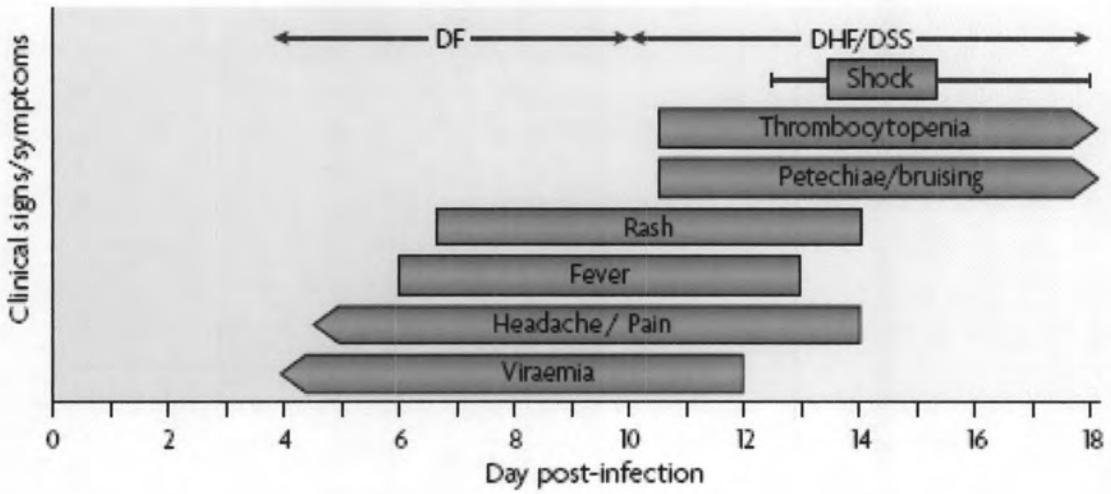


Figure. 5 Classification of symptomatic dengue virus infection.

Table 1. Time course of clinical sign and symptom.



Pathology and pathogenesis of dengue hemorrhagic fever and dengue shock syndrome

On pathological examination of fatal cases, serous effusion in the pleural and abdominal cavities and hemorrhage in the skin, subcutaneous tissue, gastrointestinal tract, heart, and other organs are almost invariably present (35, 36). Histopathological changes are observed in three major organ systems: the liver, lymphocytic tissue, and the vascular systems (38). Most changes in the liver are focal and mild and are similar to those observed in the early phase of yellow fever infection: fatty metamorphosis, the formation of Councilman Bodies, and degeneration of liver cells and Kupffer cells (42). The recovery of dengue virus from the liver of children with fatal dengue and the detection of dengue viral antigens in liver cells suggest that some of these changes may result from a direct viral infection of the liver (32,43). Changes secondary to circulatory failure, such as perisinusoidal oedema, congestion, and hemorrhage, may also be present. Lymphocytic tissue in the spleen, lymph modes, and liver shows increased activity as evidenced by both proliferation of lymphoblastoid cells and lymphocytic phagocytes, indicating considerable turnover of lymphocytes. Upon the finding of exudates in the extra-vascular compartment and serosal cavities align with hemorrhage, one would expect an endothelium severely damaged to the extent of cellular necrosis. However, light and electron microscopy of blood vessels do not show such changes in the vascular wall. Rather, a swelling of endothelial cells and diapedesis of erythrocytes through vessel walls with perivascular infiltration by lymphocytes and mononuclear cells may be observed (42, 44).

Dengue Disease Pathogenesis

DHF can occur in both primary and secondary infections. It is likely that viral virulence and immunological responses both contribute to the pathogenesis of DHF. Dengue virus strains that have an ability to grow better *in vivo* may be responsible for more severe disease. Some dengue virus strains need enhancing antibody in the pathogenesis; thus, these strains cause DHF only in the secondary infection in which enhancing antibodies are available. Early intense production of cytokines by dengue

virus-infected monocytes and activated T lymphocytes is more marked in DHF than DF. Some cytokines that are known to be elevated in patients with DHF can directly or indirectly lead to plasma leakage and shock. Complement activation occurs during the period of capillary leakage. In the secondary infection by a serotype different from that which caused the primary infection, serotype-cross-reactive, non-neutralizing antibodies increase the number of dengue virus infected monocytes via a mechanism of antibodiy-dependent enhancement. Neutralizing antibody to dengue virus play a very important role in the prevention of dengue virus infections. Non-neutralizing cross-reactive antibodies, however, markedly augment dengue virus infection of FcÝ receptor- positive cell (12).

Individual may be infected by heterologous virus serotypes those experienced more one infections have a much higher propensity of developing hemorrhagic disease. Immune enhancement that hypothesis of disease occurs as a result of antibodies formed against the first infecting virus and/or as a result of increased cytokine release by antigen-presenting cell, endothelial cell, and T cell of the immune system (figure 6) (12,45, 46, 47). Also, the genetic backgrounds of the human host or other underlying disease have been hypothesizing to increase disease pathogenesis (9, 10, 48, 49). However, there are numerous documented case of hemorrhagic dengue occur after primary infection thus also pointing to differences in viral virulence as a factor in pathogenesis. Recent studies have showed that the first targets of dengue virus infection as dendritic cells and macrophage (1,13, 50, 51) have a great influence on the amount of virus replication and circulating in patient can determine viral load that seems to be correlated with disease outcome (52, 54).

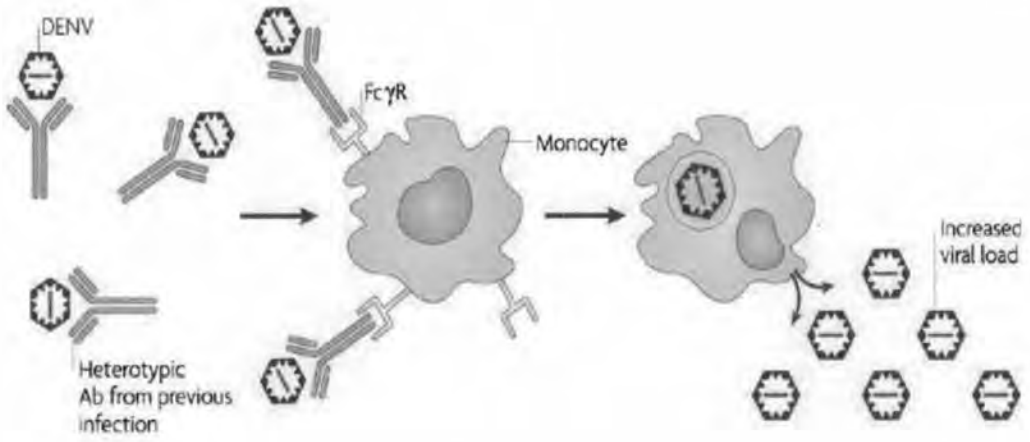


Figure 6. Antibody (Ab)-dependent enhancement of viral replication occurs when heterotypic, non-neutralizing Ab present in the host from a primary dengue virus (DENV) infection binds to an infecting DENV particle during a subsequent heterotypic infection but cannot neutralize the virus. Instead, the Ab-virus complex attaches to the Fc receptors (FcR) on circulating monocytes, thereby facilitating the infection of Fc R cell types in the body not readily in the absence of antibody. The overall outcome is an increase in the overall replication of virus, leading to the potential for more severe disease (27).

Diagnosis

Laboratory diagnosis of dengue relies on the recovery of the virus by culture or on the detection of antibodies to the dengue virus. Although culture is the definitive diagnostic test, several practical considerations limit its use. First, virus isolation is mainly successful when attempted using clinical samples obtained during the acute phase of disease. Already within a day or two after fever defervescence, antibodies to the dengue virus will begin to interfere with virus culture. Second, samples obtained for the detection of dengue virus by culture require proper handling. If storage and transportation conditions are not optimal, the virus will be inactivated in the

samples. And third, not all laboratories are capable of culturing dengue viruses due to financial and safety reasons (2).

Several promising laboratory techniques have been introduced in recent years with which either viral RNA or viral antigen can be detected in samples obtained from infected patients. Molecular diagnosis typically provides more sensitive and rapid detection than traditional virus isolation methods, because it amplifies nucleic acid even for inactivated virus. Amplification of dengue RNA by reverse transcriptase polymerase chain reaction (RT-PCR) has proved successful .Nested RT-PCR, using the primer at different regions of the dengue viral genome, has been developed for detecting and typing DENV in clinical samples. Two sets of primers corresponding to the C/prM region of the virus, designed by Lanciotti et al, are frequently used to identify DENV and it serotypes via 2-step nested RT-PCR (51). The potential diagnostic usefulness of RT-PCR assay is to detect DENV in human serum. This assay demonstrates sensitivities of 94% with DENV-1, 93% with DENV-2 and 100% with DENV-3 and DENV-4, compared with the traditional method of virus isolation (42). To minimize contamination and maximize cost effectiveness, Harris et al established a modified single tube multiplex RT-PCR (52). The detection limits of Harris' protocol were around 1 plaque-forming unit (PFU) for DENV-1, 50 PFU for DENV-2, 1 PFU for DENV-3, and 30 PFU for DENV-4. De Paula et al also showed that 1-tube RT-PCR protocols obtained a higher rate of DENV detection than the 2-step methods, and these data correlated well with serologic diagnosis (31). However, 1-tube RT-PCR is easier to use when processing large numbers of samples, and helps to minimize carry-over contamination resulting from unopened tubes between cDNA synthesis and amplification. Quantification of DENV RNA in human plasma samples can provide more clues for performing pathogenesis studies and monitoring the progress of clinical manifestations. Quantitative competitive RT-PCR at C-prM or 3'-non-coding region has been developed for determining dengue viral load, and has sensitivity of 10 to 250 RNA copies (53). Fluorogenic RT-PCR based on 3'-noncoding sequence provided an alternative quantitative method for measuring DENV RNA, and had a detection limit of 20-50 PFU/ml (54, 55). Real-time RT-PCR assay represents another choice for quantifying DENV RNA. Recently, real-time PCR assay, measuring positive as

well as negative sense RNA, was also used for detecting replicating DENV in PBMC from dengue patients, a good indicator showing that the virus in the infected cells had not been cleared by the cell-mediated immunity (56). Enzyme-linked immunosorbent assays (ELISAs) have been developed to detect the NS1 protein, and several reports indicate that detection of the NS1 protein may allow early diagnosis of infection (57). Although an antigen detection kit is commercially available, its potential diagnostic use still needs to be evaluated properly (Figure 7.)(10)

Serological assays are most widely used in routine practice to confirm dengue virus infections and to differentiate between a primary and a secondary infection (39). Primary infections are characterized by an increase in the levels of dengue-virus-specific immunoglobulin M (IgM) antibody 3-5 days after the onset of fever. IgM antibody titers continue to rise for 1-3 weeks and remain detectable for up to 6 months. Dengue-virus-specific immunoglobulin G (IgG) antibodies increase shortly after the initial rise in IgM antibodies to a modest degree and remain detectable for life. In secondary infections, the IgM antibody level is generally lower than in primary infections, while IgG antibody levels rise rapidly from 1 to 2 days after the onset of symptoms (58). The most commonly used serological techniques for the diagnosis of dengue infection are an ELISA that detects IgG or IgM antibodies and the haemagglutination-inhibition test. Traditionally, the haemagglutination-inhibition test has been used for the diagnosis of dengue and requires paired acute and convalescent sera collected 1 week or more apart for definitive diagnosis. However, this test requires samples to be pretreated, and the variability in methods used in different laboratories has compromised its general applicability. Today, many laboratories rely on other immunosystems for the diagnosis of dengue infection: ELISAs, immunochromatographic assays, or dot-blot assays (59). The most widely used are an IgM antibody-captured enzyme-linked immunosorbent assay (MAC-ELISA) or a combination of IgM and IgG by ELISA. The interpretation of serological test results may be complicated by the occurrence of cross-reactive antibodies to antigenic determinants shared by other members of the flavivirus family (i.e. Japanese encephalitis virus). Commercial kits for the rapid detection of IgG as well

as IgM antibodies have become available and seem to be useful and reliable for serodiagnosis of dengue virus infection (60).

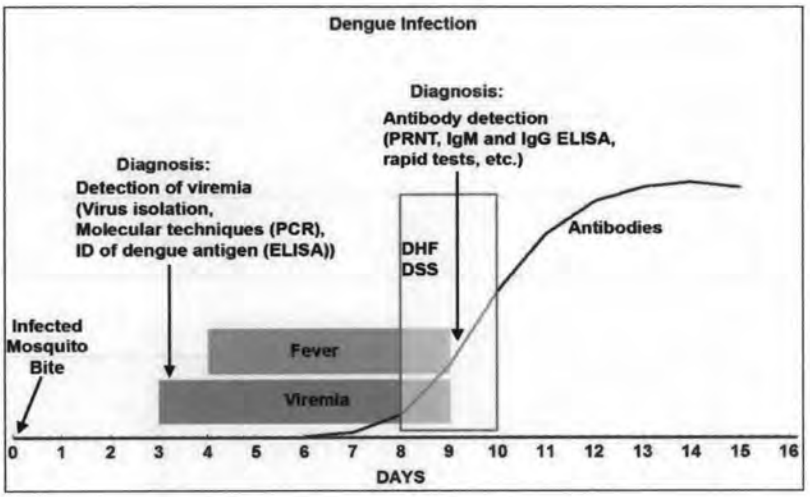


Figure 7. Course of dengue infection and timings of diagnosis.

Prevention and Treatment

Prevention

Although urgently needed, no vaccine is currently available to protect against dengue virus infections. An effective vaccine will have to provide protective immunity against all four dengue viruses, bearing in mind concerns about ADE enhancement (61). Promising result from studies with a live attenuated chimeric vaccine against Japanese encephalitis and the development of new dengue vaccines using a similar methodology provide ample basis for optimism that a safe vaccine is feasible (62). Until a vaccine is available, large dengue outbreaks can only be stopped by controlling vector populations. In addition to insecticide application, breeding sites of *Aedes* mosquitoes should be reduced vigorously or properly managed. Stagnant water should be avoided and containers of standing water should be emptied. Personal protection measures, such as the use of mosquito repellents, protective clothing, or insecticides, are necessary to protect travelers and residents of endemic areas against dengue virus infections. Effective and sustainable prevention of dengue outbreaks must include active community participation, a good public health infrastructure, and political

will. Although eradication programs may impose a financial burden on developing countries, the failure of these programs in the Americas is a good example of what can happen when political and community support is reduced. Recent education programmed has resulted in an increased awareness of the seriousness of severe dengue virus infections among residents living in dengue-endemic areas. However, such programmed did not always bring about behavioral changes or result in adequate control of mosquito habitats (63)

Treatment

Currently no specific therapeutic agent exists for dengue. Mild dengue infections may be treated with oral hydration and antipyretics only. Paracetamol should be used instead of aspirin or other nonsteroidal anti-inflammatory drugs to avoid the increased risk of Reye's syndrome and hemorrhage (64). Patients need to be monitored closely for signs of shock until at least 24 h after defervescence. For patients suffering from DSS, the mainstay of therapy is early and effective replacement of plasma loss. The World Health Organization recommends immediate volume replacement with Ringer's lactate, Ringer's acetate, or 5% glucose diluted in physiological saline, followed by plasma or colloid solutions in the event that shock persists. Recently, two randomized controlled trials evaluated therapeutic responses to colloid and crystalloid solutions (65). Results indicate that Ringer's lactate performed the least well and that the more severely ill patients, identified by a narrow pulse pressure ($\leq$10 mmHg), would benefit more from initial resuscitation with colloid solution than with crystalloid solution. However, these studies were underpowered and the findings will need to be confirmed in large studies in which patients are stratified by severity of disease on admission.

Since volume replacement therapy alone may be insufficient for those admitted late in the course of disease, additional interventions for the treatment of dengue virus infections have been sought. Carbozochrome sodium sulfonate (AC-17), drug that blocked capillary permeability in an animal model, failed to prevent dengue vascular permeability or shock in humans. High-dose methylprednisolone did not reduce mortality in severe DSS, although prolonged thrombocytopaenia following DHF

responded well to corticosteroids. In addition, several case reports described the potential use of decompressing, high dosages of immunoglobulin, and recombinant activated factor VII in dengue virus infections (66). The administration of these drugs was followed by an immediate clinical response, but clinical trials evaluating their effectiveness are still awaited.

Virulent strains of dengue

Dengue virus differs genotypically. The genotypic differences appear to be associated with the difference in virulence (67). For instance, the first large outbreak of DHF in the American occurred in Cuba in 1981. This outbreak coincided with the introduction of a new strain of dengue virus type 2 to this region. Phylogenetic studies demonstrated that this new strain was the Southeast Asian genotype of dengue virus type 2 that was different from the original American genotype of dengue virus type 2. The introduction of the Southeast Asian genotype coincided with the appearance of DHF in different countries in this region, while the original American genotype was only associated with DF, but no with DHF (68, 69). Other epidemiological studies demonstrated that there were no DHF cases reported in Peru where the Southeast Asian genotype of dengue virus type 2 was not introduced (70). There are studies that have attempted to define the molecular determinants of virulence. Rico-Hesse, et al. reported that the determinants for virulence resided at the amino acid 390 of the E protein, in the 5' nontranslated region and in the upstream 300 nucleotides of the 3' nontranslated region (71). The other group demonstrated non-synonymous amino acid replacements in the PreM, NS1, NS2a, NS3, and NS5 by analysis multiple strains of dengue virus type 2. They classified these strains into three subtypes on the basis of the severity of the original patients and amino acid replacements (72, 73, 74). The strains that may induce DSS, the strain that may induce DF in primary infection, but DHF in secondary infection, and those induce only DF in both primary and secondary infections. However further studies are needed to elucidate the molecular bases underlying these possible different phenotypes.

Dengue serotypes are co-circulating in some areas and some individuals.

M. A. Lorono-Pino, et al (75) screened viremic serum samples and mosquitoes inoculation with serum samples collected during epidemics involving multiple dengue virus serotypes in Indonesia, Mexico, and Puerto Rico for virus isolation, 16 of 292 (5.5%) samples were found to contain 2 or more dengue viruses by an indirect immunofluorescence test and reverse transcriptase-polymerase chain reaction. Next study by Phaisan Khawsak, Sirichai Phantana, and Kosum Chansiri (76) investigated dengue virus serotypes of human blood samples in Thailand during 2000-2001 by RT-PCR technique. They found that 71(32.71%), 43(19.85%), 28(12.9%), and 43 (19.85%) patients were classified as having single serotypes 1, 2, 3, and 4 respectively and they found multiple infections with two or more dengue virus serotypes. In the same year, Wei-Kung Wang et al (77) detected and determined the serotypes of 21 dengue patients during an outbreak in southern Taiwan in 2000 by multiplex reverse transcription polymerase chain reaction. 2 of 21 (9.52%) cases were concurrent infections by dengue type 2 and dengue type 3 virus. In addition, Maraiana Aparecida Antunes Bastos et al (78) studied dengue serotypes in autochthonous cases by immunofluorescence and RT-PCR. They found double infections by DEN-1 and DEN-2 which were of genotype I and genotype III, respectively. A recent study in 2006 by Preeti Bharaj et al (79) detected dengue serotypes of 48 sera samples from patients during outbreak in Delhi in 2006 by RT-PCR and ELISA. Concurrent infections of more than one dengue virus serotypes were identified in 9 of 48 cases (19%).

Dengue genotypes

Eva Harris et al (80, 81) investigated epidemiology of dengue infections in an endemic country and describe a simple, rapid, PCR-based subtyping method, restriction site-specific (RSS)-PCR. They found that dengue virus could be divided into subgroups. DEN-1 has 3 major RSS-PCR types. 7 types were observed for DEN-2, 3 types were observed for DEN-3, and 2 types were observed for DEN-4. In 2002, M. P. Miagostovich et al (62) characterized DEN-3 isolates from autochthonous cases in the

State of Rio de Janeiro, Brazil in 2001 by Restriction site-specific (RSS)-PCR. Of 22 strains of the Brazilian DEN-3 viruses classified as subtype C, these contain viruses from Sri Lanka, India, Africa and Central America. Recently, Marize P. et al (34) performed a molecular characterization of DEN-2 viruses 52 isolates in Brazil during 1990-2000 from geographically and temporally distinct areas by RSS-PCR. The RSS-PCR patterns and phylogenetic analyses showed that the studied DEN-2 isolates belonged to genotype III (Jamaica genotypes).

Dengue virus evolution.

Viral RNA-dependent RNA polymerase are of notoriously low fidelity incorporation of mutations into the progeny RNA strand, coupled with the lack of a second strand for proofreading, result in the generation of a cloud of variant viral RNA species, dubbed quasispecies. Although most often associated with chronic infection with RNA viruses, such as hepatitis C virus, it has become clear that acute RNA virus infections also result in significant intra-host sequence diversity. A work on poliovirus has shown that the degree of sequence diversity of the viral RNA genome must be carefully regulated; too many mutations lead to error catastrophe (82), while too few could result in reduced pathogenesis (83). Lately, studies focusing on the C, E, and NS2B genes have indicated that dengue virus also exhibits substantial sequence diversity in humans suggesting that intra-host sequence variation of DEN-3 is likely to reflect genetic drift. These findings would add to standing of the evolution of dengue virus in human (74) and to a lesser extent in mosquitoes (84). Mosquitoes contribute to the evolutionary conservation of dengue virus by maintaining a more homogenous viral population and a dominant variant during transmission. In 2002 it was demonstrated that the dengue virus circulated as a population of closely related genomes. The extent of sequence diversity and genome-defective viruses were found. The findings on the quasispecies nature of dengue virus and defective viruses *in vivo* have an implication on the pathogenesis of dengue virus (85). An intriguing report recently demonstrated that a defective DEN-1 lineage acquired a stop-codon mutation in the surface envelope (E) protein gene and was disseminated and maintained in human populations in

Myanmar over at least 2 years. This finding not only provides further evidence of intra-host diversity of viral species but also implies a possibility of complementation of the defective genome by co- infection of cell with functional viruses (86).