STUDY OF VON WILLEBRAND FACTOR IN PATIENTS WITH DENGUE HEMORRHAGIC FEVER

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STUDY OF VON WILLEBRAND FACTOR IN PATIENTS WITH DENGUE HEMORRHAGIC FEVER

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ABSTRACT

Von Willebrand factor (VWF), produced and released by endothelial cells and megakaryocytes, plays a critical role in the adhesion of platelets to damaged subendothelium sites of vessel injury and is a carrier for coagulation factor VIII in plasma. The abnormalities of VWF can result in hemorrhage and hemostasis disorders in patients. Knowledge of VWF in patients with dengue virus infection is not well understood.

The aim of this study is to investigate and evaluate the quantitative and qualitative aspects of VWF in dengue infected patients, the study evaluated abnormalities of VWF, including the level of VWF antigen (VWF:Ag), ristocetin cofactor activitiy (VWF:RcoF) and VWF multimer in 21 dengue fever (DF), 30 dengue hemorrhagic fever grade I (DHF I), 33 dengue hemorrhagic fever grade II (DHF II), 10 dengue shock syndrome (DSS) and 7 other febrile illness (OFIs) patients. The levels of VWF:Ag and VWF:RcoF on day 0 and day 1 were significantly higher in DSS patients than in the other groups of patients ($p < 0.05$). The levels of VWF:Ag were not significantly different among in DF, DHF I, DHF II and OFIs patients. In addition, the levels of VWF:RcoF were significantly higher in non shock DHF patients than in DF and OFIs patients ($p < 0.05$). Finally the study found that patients with DF, DHF I, DHF II and DSS have no abnormal structure VWF multimer. These findings showed that there were no decreased abnormalities in quantity and function of VWF, and neither decreased nor increased abnormalities in the structure of VWF in patients with dengue viral infection, and the levels of VWF:Ag and VWF:RcoF were correlated with disease severity.

The relative risk assessment of laboratory findings indicate that the patients with VWF:Ag and/or VWF:RcoF $\geq$ 210 % being at higher risk of contracting DSS. Therefore this may be used as a prediction of DSS in patients with dengue virus infection.

KEY WORDS: DENGUE HEMORRHAGIC FEVER/ VON WILLEBRAND FACTOR/ RISTOCETIN COFACTOR ACTIVITY/ VWF MULTIMER.

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การศึกษา VON WILLEBRAND FACTOR ในผู้ป่วยโรคไข้เลือดออก
(STUDY OF VON WILLEBRAND FACTOR IN PATIENTS WITH DENGUE HEMORRHAGIC FEVER)

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บทคัดย่อ

Von Willebrand factor (VWF) ถูกสร้างและหลั่งออกมาจากเซลล์บุผนังหลอดเลือดและเกล็ดเลือด มีบทบาทสำคัญคือเป็นตัวเชื่อมการยึดเกาะของเกล็ดเลือดกับเซลล์บุผนังหลอดเลือดชั้นใน ตรงบริเวณที่มีการฉีกขาดหรือเกิดบาดแผลของหลอดเลือด และยังเป็นพาหะของปัจจัยการแข็งตัวของเลือด factor VIII ในพลาสมา ความผิดปกติของ VWF สามารถทำให้ผู้ป่วยเกิดภาวะเลือดออกผิดปกติและมีความผิดปกติของภาวะสมดุลในระบบเลือด ความเข้าใจเกี่ยวกับ VWF ในผู้ป่วยโรคไข้เลือดออกยังไม่เป็นที่เข้าใจมากนัก เพื่อศึกษาและประเมินคุณลักษณะของ VWF ในผู้ป่วยที่ติดเชื้อไวรัสเด็งกี่ จึงได้ทำการศึกษาหาปริมาณของ VWF (VWF:Ag), คุณภาพการทำหน้าที่ของ VWF (VWF:RcoF) และโครงสร้างโมเลกุลขนาดใหญ่ของ VWF (VWF multimer) ในพลาสมาของผู้ป่วยจำนวน 101 รายประกอบด้วย ไข้เด็งกี่ จำนวน 21 ราย,ไข้เลือดออกระดับ 1 จำนวน 30 ราย, ไข้เลือดออกระดับ 2 จำนวน 33 ราย, ไข้เลือดออกที่มีภาวะช็อก จำนวน 10 ราย และผู้ป่วยที่มีไข้โรคอื่นที่ไม่ได้ติดเชื้อไวรัสเด็งกี่ จำนวน 7 ราย ผลการศึกษาพบว่า ปริมาณและคุณภาพการทำหน้าที่ของ VWF ในผู้ป่วยไข้เลือดออกที่มีภาวะช็อก ในวันที่ไข้ลดและหลังจากนั้น 1 วัน มีค่าสูงกว่าที่พบในกลุ่มอื่น ๆ ($p< 0.05$) และยังพบว่าปริมาณของ VWF ในกลุ่มผู้ป่วยไข้เด็งกี่, ไข้เลือดออกระดับ 1, ไข้เลือดออกระดับ 2 และผู้ป่วยที่มีไข้โรคอื่นที่ไม่ได้ติดเชื้อไวรัสเด็งกี่ มีค่าไม่แตกต่างกัน ($p< 0.05$) แต่คุณภาพการทำหน้าที่ของ VWF ในกลุ่มผู้ป่วย ไข้เลือดออกระดับ 1 และไข้เลือดออกระดับ 2 มีค่าสูงกว่าที่พบในผู้ป่วยไข้เด็งกี่และผู้ป่วยที่มีไข้โรคอื่นที่ไม่ได้ติดเชื้อไวรัสเด็งกี่ ($p<0.05$) และพบว่าในผู้ป่วยทุกกลุ่มมี VWF multimer ปกติ ในการประเมินภาวะเสี่ยงในการเกิดโรคพบว่า ในผู้ป่วยที่มีปริมาณ VWF:Ag และ/หรือ VWF:RcoF $\geq$ 210 % จะพบในผู้ป่วยไข้เลือดออกที่มีภาวะช็อกมากกว่าในกลุ่มอื่นๆ ($p< 0.05$)

จากผลการศึกษาดังกล่าว บ่งชี้ว่ามีการเพิ่มการสร้างปริมาณและการทำหน้าที่ของ VWF อีกทั้งไม่พบความผิดปกติของ VWF multimer ในผู้ป่วยที่ติดเชื้อไวรัสเด็งกี่ ในขณะเดียวกันยังพบว่า ปริมาณของ VWF:Ag และ VWF:RcoF ที่เพิ่มขึ้นอาจจะช่วยพยากรณ์ถึงโรคไข้เลือดออกที่มีภาวะช็อกได้

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LIST OF ABBREVIATIONS

α	=	alpha
ADAMTS 13	=	a disintegrin-like and metalloprotease with thrombospondin type 1 motif no. 13
ADE	=	antibody-dependent enhancement
APTT	=	activated partial thromboplastin time
AVWS	=	acquired von Willebrand syndrome
BCT	=	Behring Coagulation Time
β-HCG	=	beta-human chronionic gonadotrophin
BSA	=	bovine serum albumin
^{o}C	=	degree Celsius
CD	=	cluster of difference
CDC	=	The Center for Disease Control
CF	=	complement fixation
CI	=	confidence interval
DIC	=	disseminated intravascular coagulation
DF	=	dengue fever
DHF	=	dengue hemorrhagic fever
DSS	=	dengue shock syndrome
EDTA	=	ethylene diamine tetraacetic acid
ELISA	=	enzyme-linked immunosorbent assay
et al.	=	et alii (Latin), and other
g	=	gram
GI	=	gastrointestinal
GP	=	glycoprotein
HGT	=	high gelling temperature
HI	=	hemagglutination inhibition

LIST OF ABBREVIATIONS (Continued)

HMW	=	high molecular weight
hr	=	hour(s)
HRP	=	horse radish peroxidase
IFA	=	indirect fluorescent antibody
IFN-γ	=	interferon-gamma
IgG	=	immunoglobulin G
IgM	=	immunoglobulin M
IL	=	interleukin
Kb	=	kilobyte(s)
k Da	=	kilo dalton
MAC-ELISA	=	immunoglobulin M antibody capture-ELISA
min	=	minute(s)
ml	=	milliliter (10^{-3} liter)
mM	=	millimolar
NS	=	non structural protein
NT	=	neutralization test
OFIs	=	other febrile illness
PBS	=	phosphate buffer saline
PDGF	=	platelet-derived growth factor
PPP	=	platelet poor plasma
pre M	=	precursor to membrane protein
PT	=	prothrombin time
RGD	=	arginine-glycine-aspartate
RNA	=	ribonucleic acid
RR	=	relative risk
RT-PCR	=	reverse transcriptase-polymerase chain reaction
TGF-β	=	transforming growth factor-beta
TT	=	thrombin time

LIST OF ABBREVIATIONS (Continued)

TTP	=	thrombotic thrombocytopenic purpura
TNF-α	=	tumor necrosis factor-alpha
ULVWF	=	unusually large von Willebrand factor
VWD	=	von Willebrand disease
VWF	=	von Willebrand factor
VWF:Ag	=	von Willebrand factor antigen
VWF:RcoF	=	von Willebrand: Ristocetin cofactor activity
WHO	=	World Health Organization
μm	=	micrometer (s)
μl	=	microliter (10^{-6} liter)

CHAPTER 1
INTRODUCTION

Dengue viruses, a member of the family *Flaviviridae*, genus *Flavivirus*, are a single stranded RNA virus surrounded by an icosahedral nucleocapsid and covered by a lipid envelope. It's estimated that as many as 50-100 million dengue virus infections occur annually worldwide, 500,000 of which result in the severe forms, dengue hemorrhagic fever (DHF) and around 25,000 deaths principally in tropical and subtropical areas (1). Dengue viruses are transmitted to humans through the bite of infected female *Aedes* mosquitoes, principally *Aedes aegypti* and *Aedes albopictus*, and are therefore considered to be arboviruses (arthropod-borne viruses). Humans are the main amplifying host of the viruses. However, dengue virus is the most important flavivirus from the standpoint of worldwide morbidity and mortality. Dengue virus is classified into four different serotypes namely, type DEN-1, DEN-2, DEN-3 and DEN-4. Infection from any of the four serotypes of dengue virus can result in dengue fever or dengue hemorrhagic fever when heterologous infection is occurred. Dengue viruses produce a spectrum of disease in humans from asymptomatic, undifferentiated fever, classical dengue fever (DF) and Dengue hemorrhagic fever (DHF). WHO categorizes dengue hemorrhagic fever (DHF) into 4 grades depending on the condition of the infected host namely grade I, II, III and IV, grade III and IV are classified as Dengue shock syndrome (DSS).

Dengue fever (DF) is an acute debilitating self-limited febrile illness frequently presenting with headaches, bone or joint and muscular pains and rash, often accompanied by leukopenia and thrombocytopenia. More severe cases with incapacitating bone/joint pain ("break-bone fever") are common among adults. Dengue hemorrhagic fever (DHF) is characterized by four major clinical manifestations: high continuous fever for 2 to 7 days, hemorrhagic phenomena (a positive tourniquet test, petechiae, purpura, ecchymosis, epitaxis, gum bleeding and

gastrointestinal bleeding), hepatomegaly and circulatory failure (shock in severe cases). The prominent feature of DHF is its potential to develop into fetal dengue shock syndrome (DSS). The major hallmarks that determine disease severity and distinguish DHF from DF are plasma leakage due to increased vascular permeability and abnormal hemostasis (2). Dengue fever is observed more frequently during primary infections while DHF/DSS occurs with higher frequency in the secondary infectious persons who have experienced a previous dengue infection, and infants with waning levels of maternal dengue antibody. The first infection produces life-long immunity to the infecting serotype but only temporary and partial protection against the other three serotypes about 2-3 or up to 9 months, and secondary or sequential infections are possible after that (3-5).

All of four dengue serotypes can make persons occurred DHF. Studies in Thailand have shown a consistently high association between DEN-2 infection and DHF/DSS, but from 1983 onwards, DEN-3 was the predominant serotype recovered from patients with severe disease (6, 7). With these findings it has been postulated that antibody-dependent was responsible for the development of DHF (5). The association of DHF with secondary dengue infection in elder children and primary infection in infants with passive dengue antibody from their mothers led Halstead to propose the infection theory and the concept of antibody-dependent immune enhancement (ADE) (5, 8). It was suggested that during the second infection with a heterotypic dengue infection that differed from the primary one, pre-existing antibodies from the first infection that fail to neutralize would enhance viral uptake and replication in the mononuclear phagocytes. This has been shown to lead to higher virus loads both *in vitro* and in an *in vivo* primate model (6, 7). Such infected cell may then become a target of immune elimination mechanism which can trigger the production of mediators and activation of complement and the clotting cascade, and eventually produce DHF (5). To this end, others have suggested that the disease may be caused by T-cell activation; the levels of several cytokines such as TNF-α, IL-2, IL-6, IFN-γ, PAF, C3a, C5a and histamine and the synergistic effects of these mediators induce malfunction of vascular endothelial cells, which leads to plasma leakage, shock, and derangements of coagulation, which may lead to hemorrhagic manifestations (9).

At present the mediators and the precise mechanism(s) of plasma leakage and bleeding phenomena in DHF have not been clearly identified. There are many studies of vascular changes to understand that mechanism. In 1967, Bhamarapravati et al studied 100 autopsy DHF cases, microscopic studies showed swelling of capillary endothelial cells and perivascular edema in soft tissue but destruction of vascular endothelial cells was not apparent. There was no evidence of vasculitis and dengue virus antigens were not detected. There was no severe pathological changes in major organs other than serous effusion and hemorrhage, and some change in the liver in two third of the cases (10). After then Funahara et al demonstrated an interaction between platelet and endothelial cell having dengue antigen *in vitro* and suggested that some injury to the endothelial cells may allow the blood circulating in the vessel to interact with subendothelial collagen leading to the promotion of platelet aggregation and lysis resulting in thrombocytopenia (11). Recently, Avirutnan et al in 1998 found that the crossed reactive dengue antibody can activated complement on infected endothelial cells which leading to the formation of non-lytic complement complexes. Independent of this activation, dengue virus infected cells died within a few days via apoptotic cell death (12). Most recently Krishnamurti et al in 2001 studied platelet and endothelial cell activation in DF and DHF patients. The results revealed that the ratio of sP-selectin to the actual platelet counts increased in the acute phase samples with increasing disease severity. They suggest that hemorrhagic diathesis and the severe thrombocytopenia in dengue without shock are most likely due to platelet activation (the increase level of sP-selectin) and its consumption and that the endothelial cells activation may play some role (13).

From the evidences as mention above, malfunction of vascular endothelial cells may be the main factor involving in transient leakage syndrome and/or vasculopathy in dengue hemorrhagic patients. The endothelial cells function as barrier of the vessels, producing and releasing von Willebrand factor (VWF) and many mediators into circulation and the vessel walls for controlling the hemostasis system. The VWF plays an critical role in the adhesion of platelets (GP Ib/IX and GP IIb/IIIa) to damaged subendothelium (collagen) sites of vessel injury during the primary hemostasis. In addition, VWF circulates with factor VIII and protects it from rapid proteolytic degradation in the circulation (14-15).

The malfunction of endothelial cells during dengue virus infection may affect to quantitative and/or qualitative VWF production, which can result in hemorrhage and hemostasis disorders in patients with dengue virus infection. There are many syndromes that show abnormal VWF multimer (type 2 defect). They are associated with an autoimmune, lymphoid or plasma cell proliferated disorders (16-18). In addition, the patients with coronary heart disease, diabetes or preclampsia find the increased levels of VWF antigen, contributing to increased hemostatic action in plasma (hypercoagulability) (19-20). Furthermore, abnormalities of plasma VWF have been recognized to be associated with thrombotic thrombocytopenic purpura (TTP) for over 20 years. The typically TTP presents in individuals with systemic aggregation of platelets, thrombocytopenia and microangiopathy hemolysis anemia (21). The pathogenesis of TTP is the activation of vascular endothelial cells under high levels of shear stress; subsequently the unusually large VWF (ULVWF) multimers are released from Weibel-Palade body of endothelial cells and from α–granule of platelets. The ULVWF multimers induce the excessive adhesion and aggregation of platelets, cause in systemic platelet thrombi and thrombocytopenia. In patients with the defects of a VWF-cleaving protease (termed ADAMTS 13) is responsible for the presence of these ULVWF multimers. A VWF-cleaving protease in plasma normally prevents the entrance into the circulation (or persistence) of ULVWF multimers. The metalloprotease is referred to as ADAMTS 13 (a disintegrin and metalloprotease, with thrombospondin-1- like domains). It produced predominantly by hepatocytes (22-28).

Therefore, VWF may have associated with the abnormalities of dengue viral infection. VWF may have been useful to be a predictor of the severity of disease. The studies of VWF in dengue virus infection may provide new insights into the role of VWF in the pathogenesis of disease. The objective of this study is to investigate the quantitative and qualitative aspects of VWF in dengue infected patients. According to the recent report from the Department of Pedriatic, Faculty of Medicine Ramathibodi Hospital, 15 % of suspected patient of dengue infection would be negative from serological test (48), so this group of patients will be served as a control for this study.

CHAPTER 2
OBJECTIVES

1. To determine von Willebrand factor antigen (VWF:Ag), ristocetin cofactor activity (VWF:RcoF) and von Willebrand factor multimer (VWF multimer) in patients with dengue virus infection.
2. To evaluate von Willebrand factor antigen (VWF:Ag), ristocetin cofactor activity (VWF:RcoF) and von Willebrand factor multimer (VWF multimer) in patients with dengue virus infection.
3. To determine and evaluate the coagulation screening test (APTT, PT and TT) in patients with dengue virus infection.

CHAPTER 3
LITERATURE REVIEW

3.1 Natural History

3.1.1 The Viruses

There are four serotypes of dengue virus, namely DEN-1, DEN-2, DEN-3 and DEN-4. They belong to the genus *Flavivirus*, family *Flaviviridae* which include approximately 70 known pathogens for human. These include Yellow Fever Virus, Japanese Encephalitis Virus, Tick-Borne Encephalitis Virus and West Nile Virus. Dengue virus infections are a serious cause of morbidity and mortality in most tropical and subtropical areas of the world: mainly Southeast and South Asia, Central and South America and the Caribbean. The flaviviruses are relatively small (40-50 nm) and spherical with a lipid envelope **(Figure 3.1)**. The flavivirus is a positive strand RNA virus and its genome is approximately 11,000 bases long and consists of three structural and seven non-structural proteins. These three structural protein genes are located at the 5' end of the reading frame, encoding the nucleocapsid or core protein (C), a membrane-associated protein (M), an envelope protein (E) and seven non-structural (NS) protein genes are located at the 3' end. The sequence of proteins encoded is 5'-C-PrM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'**(Figure 3.2)** (1, 2). All flaviviruses have common group epitopes on the envelope protein that result in extensive cross-reactions in serologic tests especially among the four dengue viruses. Infection in human by one dengue serotype produces life-long immunity against reinfection by that same virus, but crossed protection to the other heterotypic serotypes are for short period. Persons living in an area of endemic dengue are mostly infected with two dengue serotypes during their lifetime (1, 2).

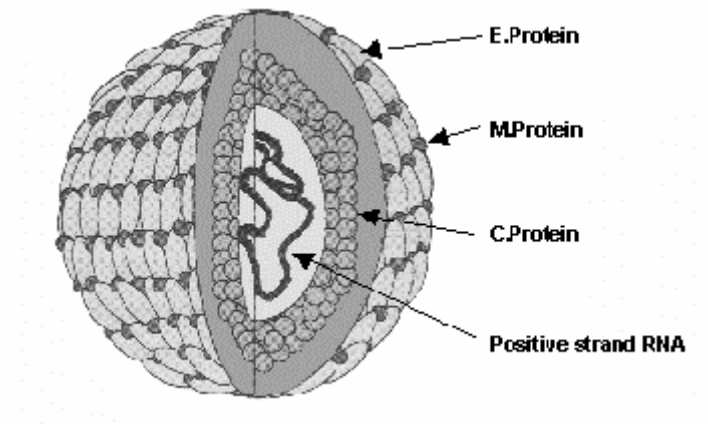


Figure 3.1 Structure of Dengue virus. (37)

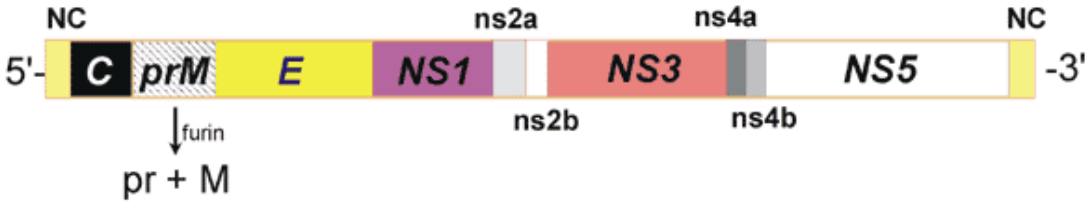


Figure 3.2 Dengue virus genome. (38)

3.1.2 Transmission of dengue viruses

The dengue life cycle involves an arthropod vector, a female *Aedes aegypti* adult mosquito and *A. albopictus* also can serve as vectors for dengue viral disease transmission. *Aedes aegypti*, the principal vector **(Figure 3.3)**, is a small, black-and-white, highly domesticated tropical mosquito that prefers to lay its eggs in artificial containers commonly found in and around homes. Humans are the main amplifying host of the virus. The adult mosquitoes prefer to rest indoors, are unobstrusive, and prefer to feed on humans during daylight hours. The female mosquitoes are very nervous feeders, disrupting the feeding process at the slightest

movement, frequently return to the same or a different person to continue feeding moments later. Because of this behavior, *A. 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 (2, 39).

Figure 3.3 *Aedes aegypti* mosquito. (40)

After an infective mosquito bites a 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 nonspecific signs and symptoms. During this acute febrile period, which may be as short as 2 days and as long as 10 days, dengue viruses may circulate in the peripheral blood (2). If other *A. aegypti* mosquitoes bite the ill person during this febrile viremic stage, those mosquitoes may become infected and subsequently transmit the virus to other uninfected persons. The virus then develops in the mosquito for a period of 8-10 days before it can be transmitted to other humans during subsequent probing and feeding **(Figure 3.4)**. The prevalence of Dengue in the world is intimately related to the mosquito population **(Figure 3.5)**.

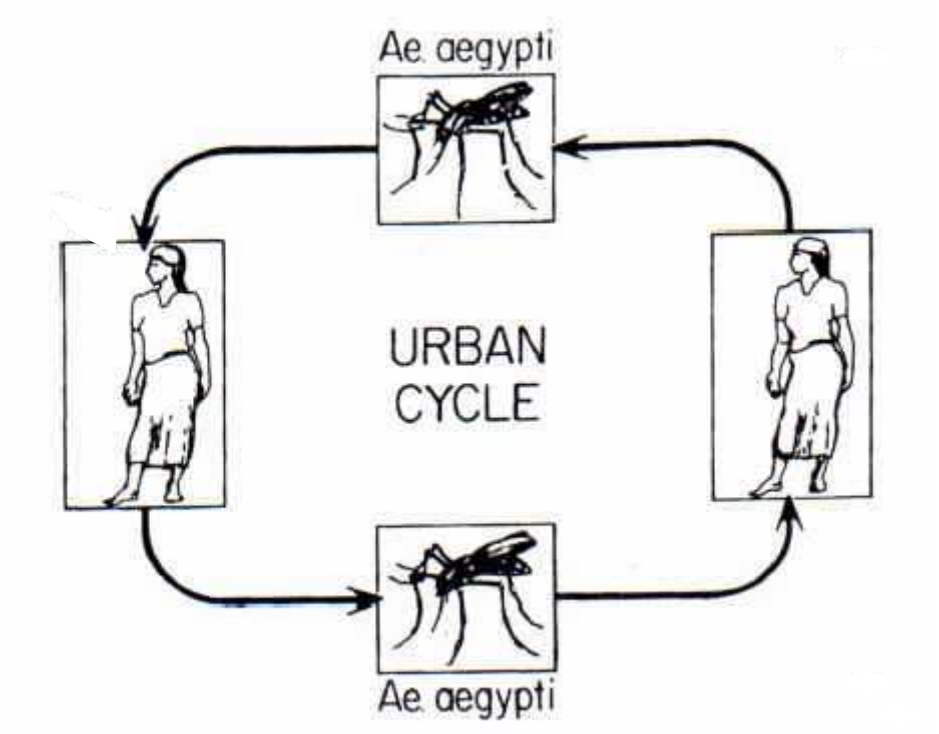


Figure 3.4 Life cycle of Dengue virus. (41)

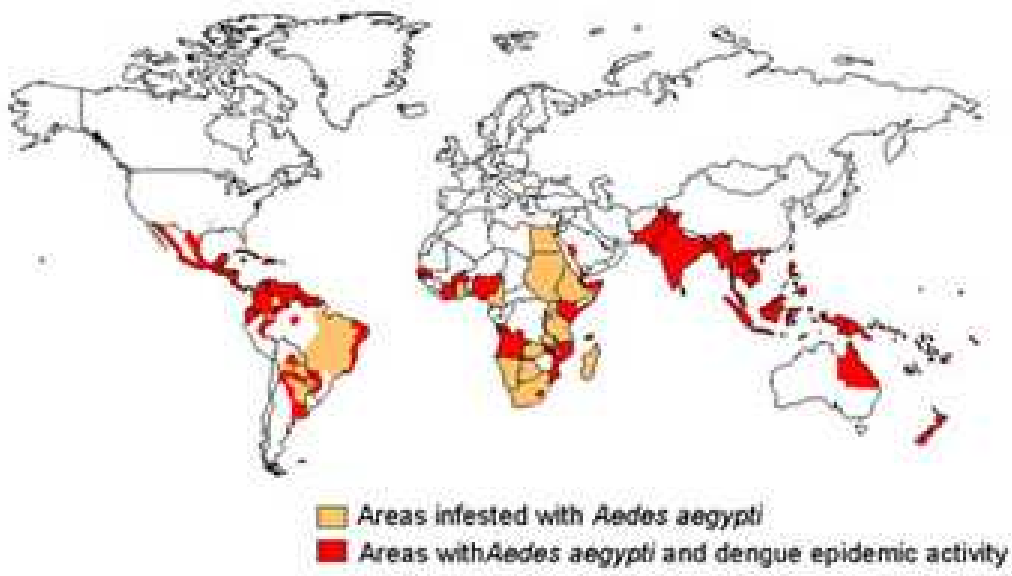


Figure 3.5 World Distribution of Dengue in 2005. (42)

3.2 Clinical Diagnosis

Dengue virus infection in humans causes a spectrum of illness ranging from asymptomatic or mild febrile illness to undifferentiated fever, DF, or DHF with plasma leakage that may lead to hypovolemic shock (DSS) **(Figure 3.6)**. Infection with any of the four serotypes causes a similar clinical symptom that may vary in severity, depending on a number of risk factors. The incubation period varies from 3 to 14 days (1, 39). In areas where dengue is endemic, the illness is often clinically non-specific, 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 (43).

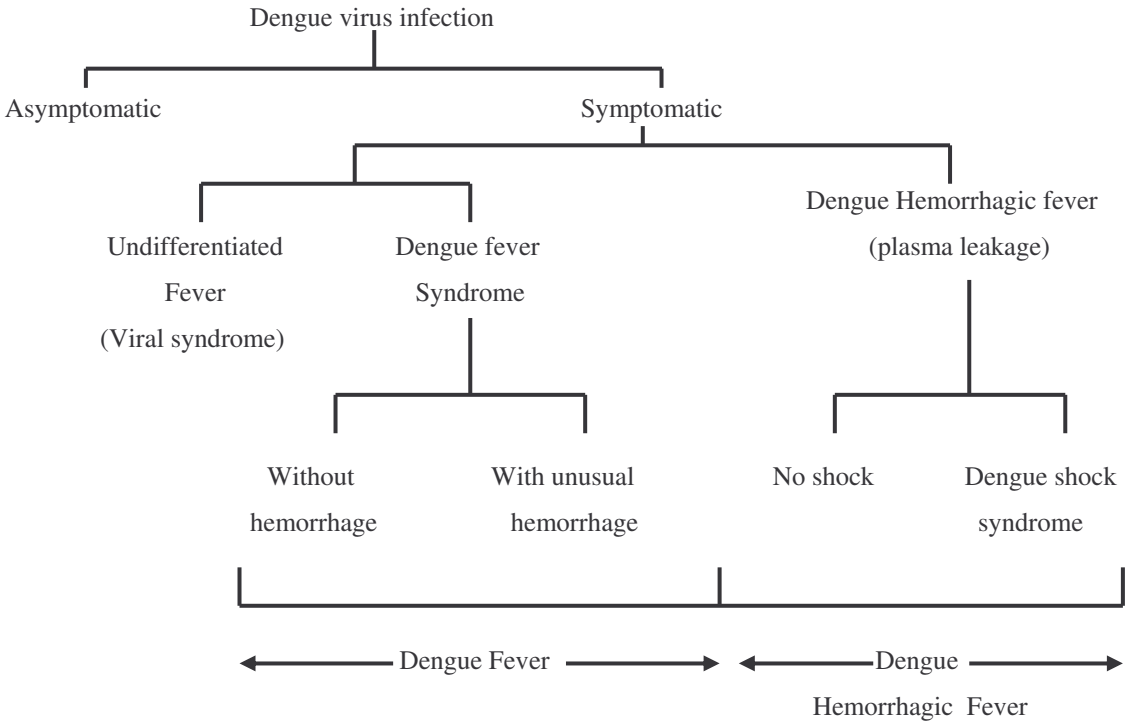


Figure 3.6 Manifestations of Dengue virus infection. (39)

3.2.1 Dengue Fever

Dengue fever is generally self-limiting and is rarely fatal. It is most common in older children and adults and characterized by the sudden onset of fever and a variety of nonspecific signs and symptoms, including frontal headache, retro-orbital pain, body aches, nausea and vomiting, joint and bone pain (break bone fever), weakness, and rash. Patients may be anorexic, have altered taste sensation, and have a mild sore throat. Lymphadenopathy, erythema or maculopapular skin rashes, occasionally with petechiae, are common. The initial temperature may rise to 39 to 41°C, and fever may last for 2 to 7 days. The fever may drop after a few days, only to rebound 12 to 24 hour's later (saddleback) (1, 2, 44).

Erythema or maculopapular skin rashes, occasionally with petechiae, are common. In rare occasions, unusual hemorrhage, mostly from gastrointestinal tract complicated DF, may result in death. Hematuria occurs infrequently, and jaundice is rare. Leukopenia is observed in patients with DF along with neutropenia followed by a lymphocytosis, often marked by atypical lymphocytes. Moderated thrombocytopenia is also common in DF and liver enzyme levels in the serum may be elevated (39, 43, 44).

3.2.2 Dengue Hemorrhagic Fever

Dengue hemorrhagic fever is a primarily disease of children under the age of 15 years, although it may also occur in adults. It has sudden onset of fever and a variety of non-specific signs and symptoms. During the acute phase of illness, it is difficult to distinguish DHF from dengue fever and other illness found in tropical areas (2). The disease is characterized by four major clinical manifestations presented below in order of their appearance and frequency (43):

- High continuous fever for 2-7 days in most cases
- Hemorrhagic diathesis most frequently presenting as skin petechiae including a positive tourniquet test
- Hepatomegaly
- Circulatory disturbances presenting as shock in severe cases

Thrombocytopenia ($\leq$100,000/mm^3) and hemoconcentration (rising hematocrit $\geq$ 20%) that representing the pathophysiologic hallmarks of abnormal hemostasis and plasma leakage respectively, are constants finding. The clinical course of DHF, unlike DF, is rather stereotypic so it is possible to make early and accurate clinical diagnosis based on the above 4 major manifestations together with the two essential laboratory finding. A normal white blood count or leukopenia is common and neutrophils, may be predominate initially. Towards the end of febrile phase there are reduction in the number of total leukocytes and neutrophils shortly before or simultaneously with relative increase in lymphocytes with the presence of atypical lymphocyte (43, 44). The liver is usually palpable early in the febrile phase. Jaundice is not usually observed. Splenomegaly is rarely observed in infants (45), mildly elevated in liver enzymes (46) and clotting abnormalities are usually found (47, 48).

The critical stage of the disease course is reached at the end of the febrile phase. Accompanying or shortly after rapid drop in temperature, varying degrees of circulatory disturbance develop (39, 43). The patient in often sweat, be restless, has cool extremities. In mild DHF cases, (WHO grades I and II) the changes in vital signs are minimal and transient. Patients recover spontaneously or shortly after a brief period of treatment. The onset of shock is acute and generally occurs at the time of defervescence. The temperature is often subnormal, the skin is cold and clammy, and the pulse becomes rapid and weak. The pulse pressure is narrow (<20mmHg) in the early stage of shock (WHO grade III). It is noteworthy that patients who are in shock usually remain conscious almost to the terminal stage. The course of shock is short but life-threatening. If the proper management is not given, the patient deteriorates rapidly into the stage of profound shock, and pulse and/or blood pressure become undetectable (WHO grade IV) (39, 43). Prolonged shock is often complicated with metabolic acidosis that may precipitate the occurrence of disseminated intravascular coagulation (DIC), or enhance the ongoing DIC to the point that massive bleeding occur. The causes of death are still mainly prolonged shock and massive bleeding (43, 45) **(Figure 3.7)**.

The World Health Organization (WHO) catagorises DHF into four grades from less severe (grade I) to severe (grade IV). Grades III and IV are also preferred to as DSS (49).

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 manifestation of Grade I patients, usually in the forms of skin or other hemorrhage.

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.

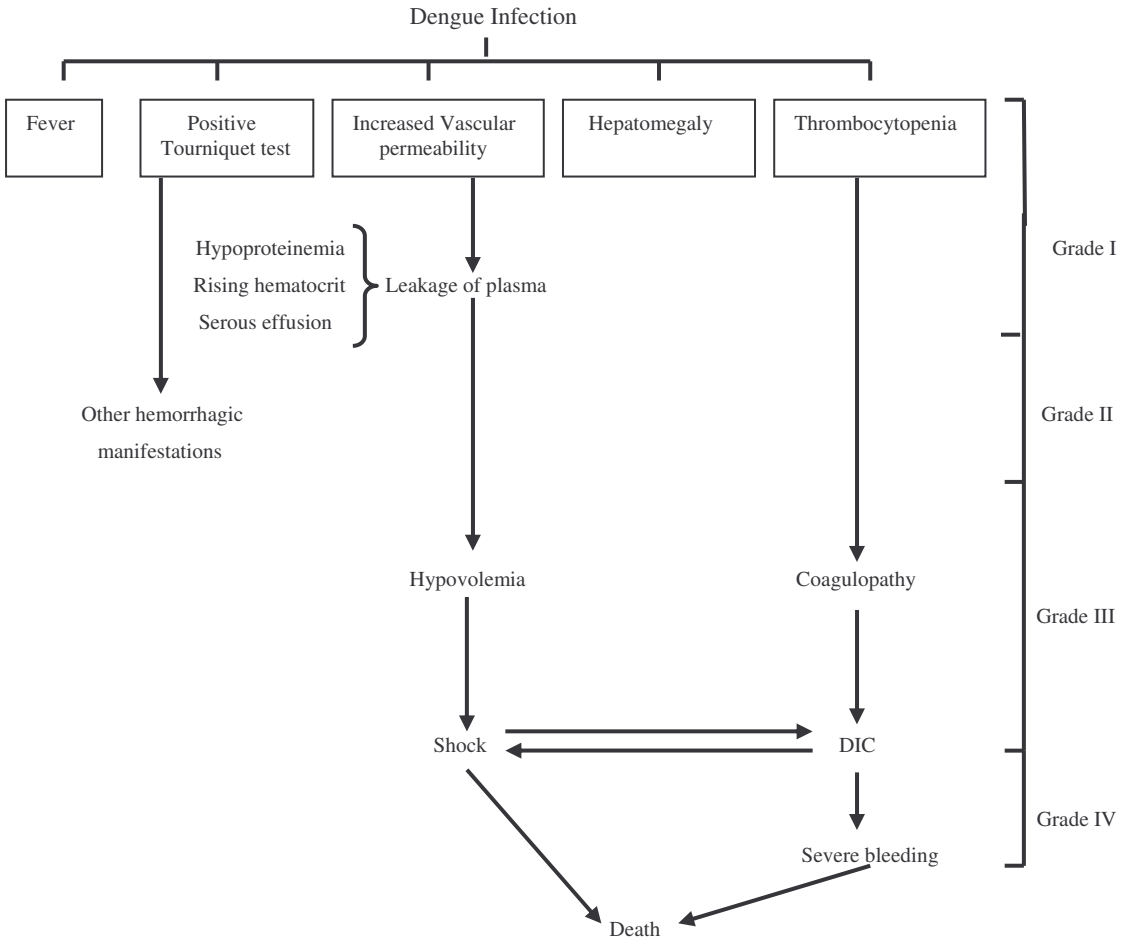


Figure 3.7 the Spectrum of dengue hemorrhagic fever. (39)

3.3 Laboratory Diagnosis

Considering the technology currently used for the diagnosis of dengue viruses, a case definition in which laboratory confirmation is emphasized and has been proposed. The laboratory criteria for confirmation of the infection and the disease include the isolation of dengue virus from serum and/or autopsy samples, the demonstration of the fourfold or greater increase in the titer of IgG or IgM antibody to one or more dengue virus antigens in paired serum samples, the demonstration of dengue virus antigen in autopsy tissue or serum samples by immunohistochemistry or by immunofluorescence and by the detection of the viral nucleic acid (39, 50).

3.3.1 Viral isolation

Four isolation systems have routinely been used for dengue viruses; intracerebral inoculation of 1- to 3-day old baby mice, the use of mammalian cell cultures (LLC-MK_2 cells), intrathoracic inoculation of adult mosquitoes, and the use of mosquito cell cultures (C6/36 cells)(2, 49). The method selected for virus isolation depends upon the laboratory facilities available. Because the mosquito inoculation technique is the most sensitive, it is the method of choice for fatal cases or patients with severe hemorrhagic disease. Use of the mosquito cell lines is the method of choice for routine virologic surveillance. Even though cell cultures are less sensitive than mosquito inoculation, this disadvantage is more than offset by the ease with which large numbers of samples can be processed in a relatively short time (2).

3.3.2 Virus Identification

The method of choice for dengue virus identification is indirect fluorescent-antibody (IFA) with stereotype-specific monoclonal antibodies produced in tissue culture or mouse ascitic fluids and an anti-mouse IgG-fluorescein isothiocyanate conjugate (51). This test can be easily performed with infected cell cultures, mosquito brain or tissue squashes, mouse brain squashes, or even on formalin-fixed tissues embedded in paraffin and sectioned for histopathologic testing.

It is simple and reliable and is the most rapid method. Moreover, it allows the detection of multiple viruses in patients with concurrent infections with more than one serotype (2, 49).

3.3.3 RT-PCR

Reverse transcriptase PCR (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 method is rapid, sensitive, simple, and reproducible if properly controlled and can be used to detect viral RNA in human clinical samples, autopsy tissues, or mosquitoes (2, 52). Several laboratories have published RT-PCR amplification protocols to detect dengue viremia. These methods feature two strategies for identification of the four dengue serotypes: combination of the four serotype-specific oligonucleotide primer pairs in a single reaction tube, or use of a universal dengue oligonucleotide primer pair, which requires a subsequent step to classify positives with serotype-specific oligonucleotides (39). Since RT-PCR is highly sensitive to amplicon contamination, without proper controls false-positive results may occur. Improvements in this technology, however, should make it even more useful in the future (49, 52).

3.3.4 Serological Diagnosis

Two patterns of serological response can be observed in acute dengue infection: primary and secondary. A primary response is seen in individuals who are not immune to flaviviruses. A secondary seroresponse pattern occurs in an individual with an acute dengue virus infection who has had a previous flavivirus infection. An individual infected with one serotype can never become infected with the same serotype. In primary dengue infection, the antibody titer rises slowly and is relatively serotype specific, although convalescent-phase sera usually contain detectable cross-reactive antibodies in low titer. In secondary infections, the antibody titer rises rapidly to high level. Frequently, even acute-phase sera show high antibody titers. The existence of cross-reactive antigenic determinants shared by all four dengue virus

serotypes and some other flaviviruses complicate the serological diagnosis of dengue viruses (49).

Five basic serologic tests have been routinely used for diagnosis of dengue infection; hemagglutination-inhibition (HI), complement fixation (CF), neutralization test (NT), IgM capture enzyme-linked immunosorbent assay (MAC-ELISA), and indirect IgG ELISA. In this study, the diagnosis of dengue infection was confirmed by the dengue-specific IgM and IgG determined by enzyme-linked immunosorbent assay (ELISA) in the acute and convalescent sera. Dengue infections were defined as secondary when the ratio of dengue-specific IgG to IgM serum antibodies was more than 1.8 (2).

Among all tests as mention above, HI has been the most frequently used as it is sensitive, 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 (titer of 10) by day 5 or 6 of illness, and antibody titers in convalescent- phase serum specimens are generally at or below 640 in primary infections. By contrast, there is an immediate anamnestic response in secondary dengue infections, reciprocal antibody titers increase rapidly during the first few days of illness and often reaching 5,120 to 10,240 or more. 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 major disadvantage of the HI test is its lack of specificity, which generally makes it unreliable for identifying the infecting virus serotype (2, 49).

The complement fixation (CF) test is not widely used for routine dengue diagnostic serologic testing. It is more difficult to perform, requires highly trained personnel, and therefore is not used in most dengue laboratories. CF antibodies generally appear later than HI antibodies but are more specific in primary infections, and usually persist for short periods. However, it is a valuable test to have in a diagnostic laboratory because of the late appearance of CF antibodies; thus some patients show a diagnostic rise in antibody titers by CF but have only stable antibody titers by HI or ELISA (2).

The neutralization test (NT) 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 (2, 49).

The IgM capture ELISA (MAC-ELISA) has become the most widely used serologic test for dengue diagnosis in the past few years. It is simple, rapid test that requires very little sophisticated equipment. Anti-dengue IgM antibody develops a little faster than IgG antibody (53). IgM antibody titers in primary infection are significantly higher than in secondary infections. MAC-ELISA with a single acute-phase serum sample is slightly less sensitive than the HI test with paired serum samples for diagnosing dengue infection. The specificity of MAC-ELISA is similar to that of HI. In both primary and secondary dengue infections, some monotypic responses may be observed, but in general, the response is broadly reactive among both dengue virus and other flavivirus antigens. 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. In areas where dengue is endemic, MAC-ELISA can be used as an inexpensive way to screen large numbers of serum specimens with relatively little effort. It is especially useful for hospitalized patients, who are generally admitted late in the illness after detectable IgM is present in the blood, but it must be emphasized again that this test should not be used for decision making related to patients management (2, 39).

An indirect IgG-ELISA has been developed that is comparable to the HI test and can also be used to differentiate primary and secondary dengue infections (54). The test is simple and easy to perform and is thus useful for high-volume testing. The IgG-ELISA is very nonspecific and exhibits the same broad cross-reactivity among flaviviruses as the HI test does; therefore, it cannot be used to identify the infecting dengue virus serotypes. However, it has a slightly higher sensitivity than the 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 (2).

3.4 Pathology

At autopsy, all patients who have died of DHF show some degree of hemorrhage; in order of frequency, hemorrhage is found in the skin and subcutaneous tissue, in the mucosa of the gastrointestinal tract, and in the heart and liver. Gastrointestinal hemorrhage may be severe, but subarachnoid or cerebral hemorrhage is rarely seen. Serous effusion with a high protein content (mostly albumin) is commonly present in the pleural and abdominal cavities, but is less common in the pericardial cavity (39).

Microscopically, perivascular edema and loss of integrity of endothelial junctions are found. Dengue antigen can be demonstrated in endothelial cells, but there is no apparent damage to the blood vessels or endothelial cells (55, 56). Capillaries and venules in the affected organ systems may show extravascular bleeding by diapedesis and perivascular hemorrhage, with perivascular infiltration by lymphocytes and mononuclear cells. Morphological evidence of intravascular clot formation in small vessels has been recognized in patients with severe hemorrhage. In most fatal cases, lymphocyte tissues shows an increased activity of the B-lymphocyte system, with active proliferation of plasma cells and lymphoblastoid cells, and active germinal centers. There is evidence indicating that proliferation of large immunoblasts and considerable turnover of the lymphocytes occurs. The latter is manifested by a reduction of white splenic pulps, lymphocytosis, and marked lymphocytic phagocytosis (39).

In the liver, there is focal necrosis of hepatic cells, swelling appearance of Councilman bodies and hyaline necrosis of Kupffer cells. Proliferation of mononuclear leukocytes, and less frequently polymorphonuclear leukocytes, occurs in the sinusoids and occasionally in the portal areas. In the brain, edema and hemorrhage have been observed but pathologic changes associated with encephalitis have not been reported (56). At autopsy, dengue virus antigen has been found predominantly in liver, spleen, thymus, lymph nodes and lung cells. The virus has also been isolated at autopsy from the bone marrow, brain, heart, kidney, liver, lungs, lymph nodes and the gastrointestinal tract (39).

3.5 Pathogenesis

The pathogenesis of DHF and DSS is still controversial. Two theories, which are not mutually exclusive, are frequently cited to explain the pathogenetic changes that occur in DHF and DSS. The most commonly accepted is known as the secondary infection or immune enhancement hypothesis (2, 5). The other hypothesis assumes that dengue viruses, like all animal viruses, vary and change genetically as a result of selection pressures as they replicate in humans and/or mosquitoes and that there are some virus strains that have greater epidemic potential. Phenotypic expression of genetic changes in the virus genome may include increased virus replication and viremia, severity of disease (virulence) and epidemic potential (2, 57, 58).

3.5.1 Antibody-dependent enhancement of dengue virus infection

This hypothesis implies that patients experiencing a secondary infection with a heterologous dengue virus serotype have a significantly higher risk for developing DHF and DSS (5). 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, however, the virus is not neutralized and is free to replicate once inside the macrophage. 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 secrete vasoactive mediators in response to dengue infection, which causes increased vascular permeability leading to hypovolemia and shock (59-64).

3.5.2 Virological factors

The genome of dengue viruses consists of a single-stranded RNA nearly 11 Kb in length and it is a plus-stranded and infectious. Dengue virus genome codes for three structural proteins, the capsid (C) protein, preM, the precursor to the membrane (M) protein, the envelope (E) protein and seven nonstructural proteins, the NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The RNA genome is included in the icosahedral capsid, and the nucleocapsid is surrounded by a lipid bilayer containing the E and M proteins (2, 39).

Dengue viruses differ genotypically. The genotypic differences appear to be associated with the difference in virulence (64, 65). For instance, the first large outbreak of DHF occurred in Cuba in 1981. This outbreak coincided with the introduction of the 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 regions, while the original American genotype was only associated with DF, but not with DHF (66-68). 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 (69).

There are many 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 (57). The other group demonstrated nonsynonymous amino acid replacements in the PreM, NS1, NS2A, NS3 and NS5 by analyzing 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 (70-73): the strains inducing DSS, the strains inducing DF in primary infection but DHF in the secondary infection and those inducing only DF in both primary and secondary infections.

DHF can occur in both the 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 available. Early intense production of cytokines by dengue virus-infected monocytes and activated T lymphocytes is more marked in DHF than DF (74). 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 which a serotype is different from that caused the primary infection, serotype-cross-reactive, non-neutralizing antibodies increase the number of dengue virus-infected monocytes via a mechanism of antibody-dependent enhancement. In addition, serotype-cross-reactive $CD8^+$ and $CD4^+$ T lymphocytes are activated and produce high levels of lymphokines (75, 76). An increase in the number of dengue virus-infected monocytes and augmented expression of HLA class I and class II by IFN-γ facilitates the recognition of the epitopes on infected cells by virus-specific T lymphocytes and result in very high levels of $CD8^+$ and $CD4^+$ T cell activation. The marked T cell activation and monocyte activation, in turn, result in the production of much higher levels of cytokines (77, 78). IFN-γ-activated monocytes may release various cytokines upon infection with dengue viruses. Once multiple cytokines are produced, a complex network of cytokines further induces production of other cytokines. This cascade eventually results in high levels of cytokines during a short period of time in DHF patients.

In summary, rapid increase in levels of cytokines and chemical mediators induces malfunction of vascular endothelial cells leading to plasma leakage and shock. It is likely that the entire process is initiated by infection with a so-called virulent dengue virus, often with the help of enhancing antibodies in secondary infection and then triggered by rapidly elevated cytokines and chemical mediators produced by monocytes and cross-reactive T lymphocytes **(Table 3.1)**.

Table 3.1 Major factors that are considered to contribute to the pathogenesis of DHF (63)

1. Dengue viruses
   i. Virulent virus strains that grow well in the absence of enhancing antibody
   ii. Virulent virus strains that grow well in the presence of enhancing antibody
2. Infection-enhancing antibody (usually serotype cross-reactive, non-neutralizing)
   i. Enhance dengue virus infection by developing virus-antibody immune complex
   ii. Activate complement
3. Monocytes/ macrophages and dendritic cells
   i. Propagate dengue virus
   ii. Produce cytokines
4. T lymphocytes (mainly serotype cross-reactive $CD4^+$ and $CD8^+$ T lymphocytes)
   i. Produce lymphokines (cytokines)
   ii. Lyse dengue virus-infected cells
5. Complement
   i. Activation products induce plasma leakage
6. Platelet decrease
   i. Plays a main role in hemorrhagic manifestation
7. Endothelial cells
   i. Play a main role in plasma leakage
   ii. Produce cytokines
8. Genetic factors
   i. HLA determines the level of immune responses
   ii. Race and other undefined genetic factors

3.6 Von Willebrand factor

Von Willebrand factor (VWF) is a large multimeric glycoprotein present in blood plasma and produced constitutively in endothelium (in the Weibel-Palade bodies), megakaryocytes (α-granules of platelets), and subendothelial connective tissue. The basic VWF monomer is a 2,050 amino acid protein. Every monomer contains a number of specific domains with a specific function; elements of note are: the D'/D3 domain, which binds to Factor VIII. The A1 domain, which binds to platelet gp1b-receptor, heparin and possibly collagen. The A3 domain, which binds to collagen. The C1 domain, in which the RGD domain binds to platelet integrin $\alpha_{IIb}\beta_3$ when this is activated. The "cysteine knot" domain (at the C-terminal end of the protein), which VWF shares with platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and β-human chorionic gonadotrophin (β-HCG, of pregnancy test fame) (79).

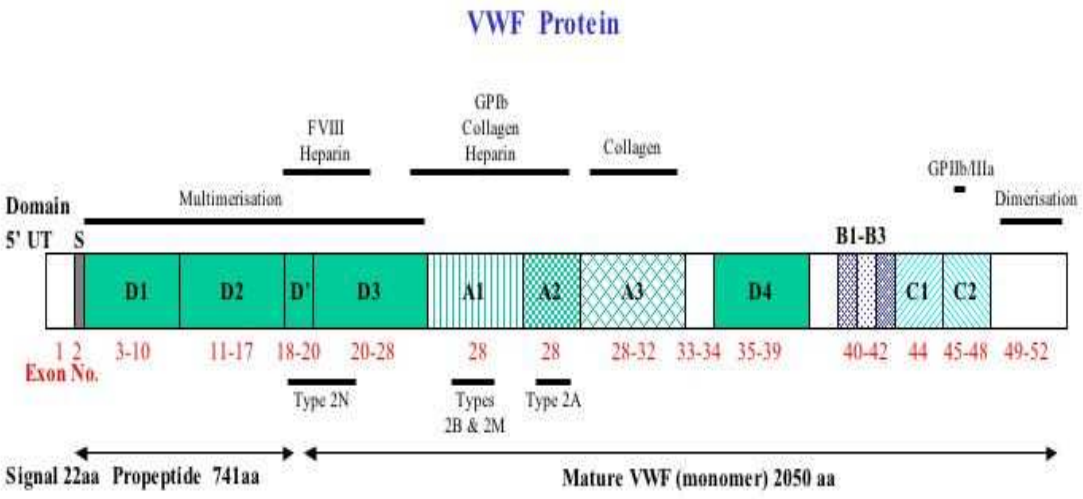


Figure 3.8 Structure of von Willebrand factor. (79)

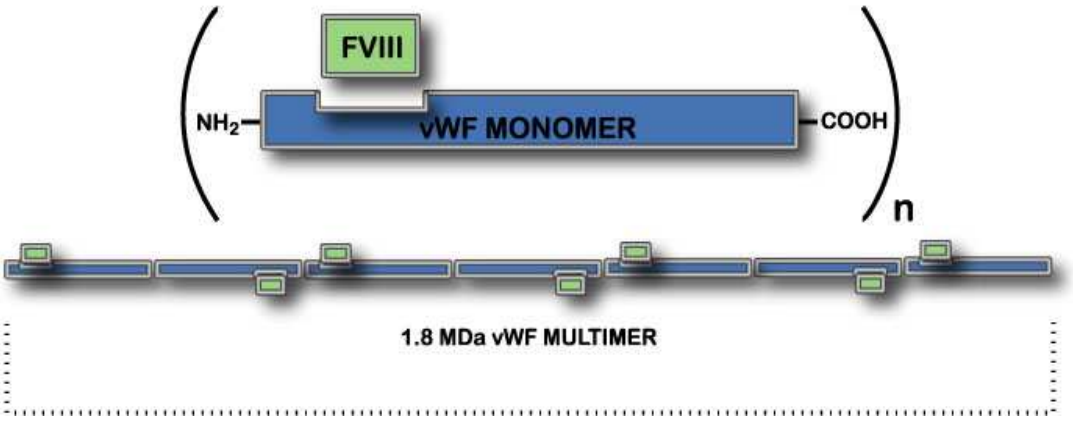


Figure 3.9 Structure of VWF multimer. (79)

Monomers are subsequently N-glycosylated, arranged into dimers in the endoplasmic reticulum and into multimers in the Golgi apparatus by crosslinking of cysteine residues via disulfide bonds. With respect to the glycosylation, VWF is one of the few proteins that carry blood group antigens (ABO system). Multimers of VWF can be extremely large, >20,000 kDa, and consist of over 80 subunits of 250 kDa each. Only the large multimers are functional. Some cleavage products that result from VWF production are also secreted but probably serve no function (79).

VWF is not an enzyme and therefore has no catalytic activity. Its primary function is binding to other proteins, particularly factor VIII and it is important in platelet adhesion to injury sites. VWF binds to a number of cells and molecules. The most important ones are; factor VIII is bound to VWF whilst inactive in circulation, factor VIII degrades rapidly when not bound to VWF. Factor VIII is released from VWF by the action of thrombin. VWF binds to collagen, e.g., when it is exposed in endothelial cells due to damage occurring to the blood vessel. VWF binds to platelet gpIb when it forms a complex with gpIX and gpV; this binding occurs under all circumstances, but is most efficient under high shear stress (i.e., rapid blood flow in narrow blood vessels, see below). VWF binds to other platelet receptors when they are activated, e.g., by thrombin (i.e., when coagulation has been stimulated). VWF appears to play a major role blood coagulation, and VWF deficiency or dysfunction (von Willebrand disease) therefore leads to a bleeding tendency, which is

most apparent in tissues having high blood flow shear in narrow vessels. From studies it appears that VWF uncoils under these circumstances, decelerating passing platelets.

The biological breakdown (catabolism) of VWF is largely mediated by a protein cryptically termed ADAMTS13 (acronym of "*a d*isintegrin-like *a*nd *m*etalloprotease with *t*hrombo*s*pondin type 1 motif no. *13*"). It is a metalloproteinase which cleaves VWF between tyrosine at position 842 and methionine at position 843 (or 1605-1606 of the gene). This breaks down the multimers into smaller units, which are degraded by other peptidases (79). Hereditary defects of VWF lead to von Willebrand disease (VWD), a bleeding diathesis of the skin and mucous membranes, causing nosebleeds, menorrhagia, and gastrointestinal bleeding. The point at which the mutation occurs determines the severity of the bleeding diathesis. Some diseases affect the structure of VWF and lead to *acquired VWD*. Recently, Heyde's syndrome (bleeding from angiodysplasia in the colon in association with aortic valve stenosis) was shown to be due to breakdown of VWF high-molecular weight multimers. In thrombotic thrombocytopenic purpura (TTP), ADAMTS13 is either deficient or has been inhibited by antibodies directed at the enzyme. This leads to decreased breakdown of the ultra large multimers of VWF and microangiopathic hemolytic anemia with severe vascular symptoms.

3.7 The acquired von Willebrand syndrome (AVWS)

The acquired von Willebrand syndrome (AVWS) is a rare bleeding disorder with laboratory findings similar to those for congenital von Willebrand disease. Unlike the congenital form, the syndrome usually occurs in individuals with no personal or family history of bleeding. Large studies on this syndrome are not available, diagnosis is still difficult and treatment empirical. Data in the literature and from a previous retrospective international registry (2000) indicate that the syndrome is especially frequent in lympho- or myeloproliferative disorders, so that it should be suspected and diagnosed with the appropriate laboratory tests when there is excessive bleeding in patients with these hematological disorders. AVWS is also associated with solid tumors, immunological and cardiovascular disorders as well as other

miscellaneous conditions. However, no prospective studies have been organized in these clinical conditions. Diagnosis of the AVWS is based on assays measuring ristocetin cofactor activity or collagen binding, which are usually abnormally low, while factor VIII coagulant activity is sometimes normal. FVIII/VWF inhibiting activities are found in only a minority of cases, but better tests to measure anti-FVIII/VWF autoantibodies are needed to screen patients with AVWS (80, 81, 82).

Pathogenetic mechanisms causing acquired von Willebrand Syndrome have the three main pathogenetic mechanisms. In the lower panel of the figure, the normal VWF biosynthesis and release from the endothelial cells is described: note that all the high molecular weight multimers (HMV VWF) are present in the released VWF after the cleavage of VWF pro-peptide (Pro-peptide of VWF) that can be found in circulation in equal amounts as native VWF. In the middle part of the figure, the three main mechanisms causing AVWS are described: a) specific or nonspecific auto-antibodies that inactivate VWF. These auto-antibodies form circulating immune complexes with VWF and are cleared, together with VWF, by Fc-bearing cells; b) absorption of VWF onto malignant cell clones; c) loss of high VWF multimers under conditions of high shear stress occurring in several heart valves abnormalities. In the upper part of the figure, the effects of these three mechanisms on VWF structure and function are described: in case of auto-antibodies (mechanism a), the entire native.

VWF normally secreted from the endothelial cells is usually removed from the circulation: this results in very low concentrations of both VWF activities and antigen but normal levels of VWF pro-peptide; when malignant cell clones (mechanism b) and conditions of high shear stress (mechanism c) cause AVWS, a preferential removal of high molecular weight (HMW) VWF multimers occurs: this results in reduced VWF activities with relatively normal VWF concentrations (80, 83, 84).

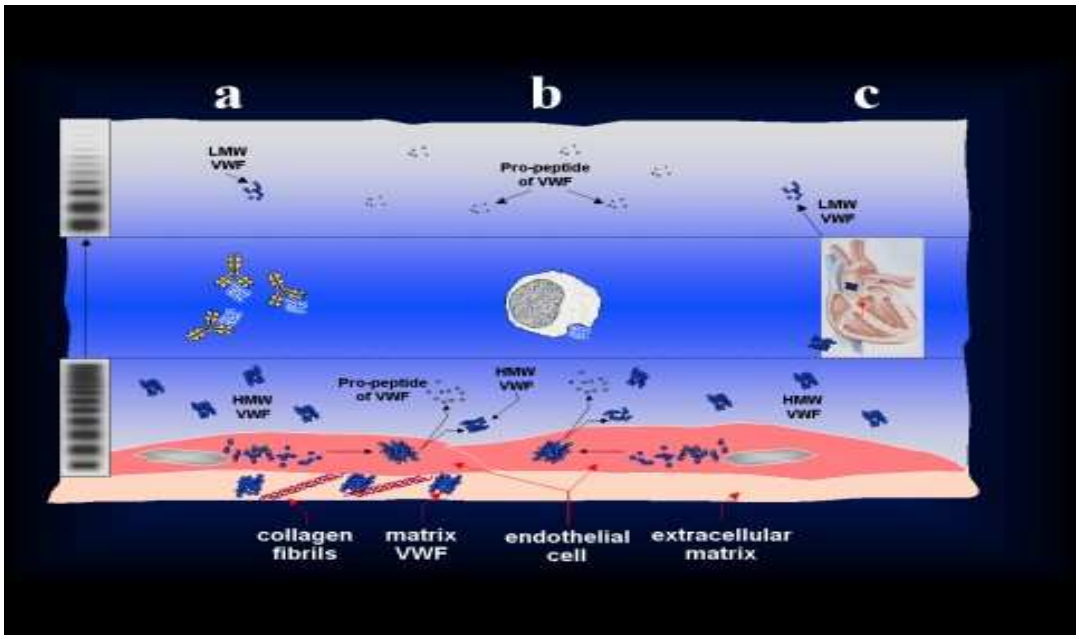


Figure 3.10 Pathogenetic mechanisms causing acquired von Willebrand Syndrome (81).

3.8 Thrombotic thrombocytopenic purpura (TTP)

Abnormalities of plasma VWF have been recognized to be associated with thrombotic thrombocytopenic purpura (TTP) for over 20 years, typically TTP presents in individuals with systemic aggregation of platelets, thrombocytopenia and microangiopathy hemolysis anemia (21). The pathogenesis of TTP is the activation vascular endothelial cells under high levels of shear stress, subsequently the unusually large VWF (ULVWF) multimers are released from Weibel-Palade body of endothelial cells and from α−granule of platelets. The ULVWF multimers induce the excessive adhesion and aggregation of platelets, cause in systemic platelet thrombi and thrombocytopenia. In patients with the defects of a VWF-cleaving protease (termed ADAMTS 13) is responsible for the presence of these ULVWF multimers. A VWF-cleaving protease in plasma normally prevents the entrance into the circulation (or persistence) of ULVWF multimers. The metalloprotease is referred to as ADAMTS 13 (a disintegrin *a*nd metalloprotease with thrombospondin type 1 motif no. 13). It

produced predominantly by hepatocytes (22-28). The defects of ADAMTS 13 is caused by; mutations in the gene for ADAMTS 13, autoantibodies against ADAMTS 13, autoantibidies against thrombospondin receptors on surface of ADAMTS13 (defective attachment of ADAMTS 13 to endothelial cells), transient defect in production or survival of ADAMTS 13 (29, 30).

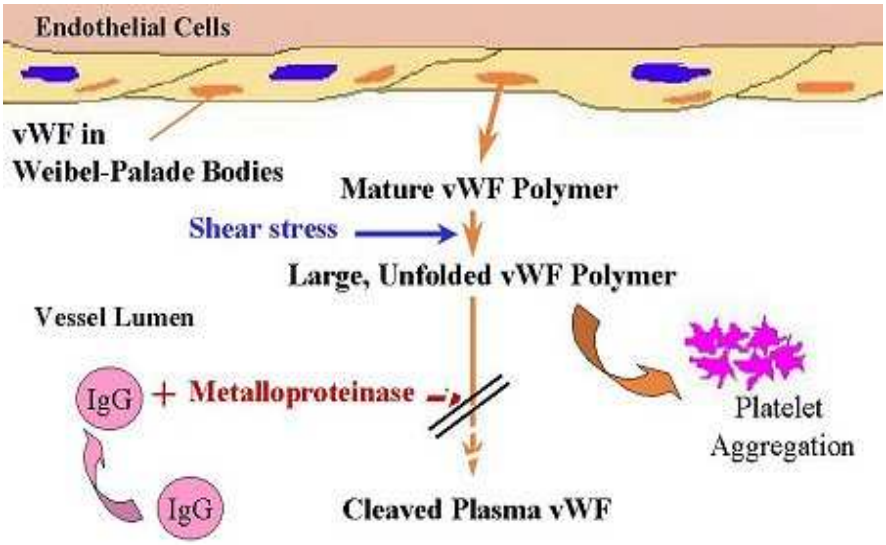


Figure 3.11 Pathogenetic mechanisms causing thrombotic thrombocytopenic purpura

Therefore, VWF may have associated with the abnormalities of dengue viral infection. VWF may have been useful to be a predictor of the severity of disease. The studies of VWF in dengue virus infection may provide new insights into the role of VWF in the pathogenesis of disease.

CHAPTER 4
MATERIALS AND METHODS

4.1 Materials

4.1.1. Instruments

1. Technicon H*3 hematology analyzer (Bayer Diagnostics, USA)
2. Refrigerator 4°C (Hitachi R-33 NCX, Japan)
3. Freezer –152°C (Sanyo Ultra low, Japan)
4. Refrigerator centrifuge (Beckman C5-15R, USA)
5. Semi-automated Coagulation Analyzer (Sysmex CA-50, Japan)
6. Autoclave (Tomy SS-320, Japan)
7. Hot air oven (Venticell 55, Germany)
8. Centrifuge (Kubota 5100, Japan)
9. pH meter (Beckman, USA)
10. Automated microplate reader (Bio-Tek, Elx 808UV, USA)
11. Power supply (Multidrive XL, Pharmacia, LKB, USA)
12. Electrophoresis chamber (2117 Multiphor II Electrophoresis Unit, Pharmacia, LKB Biotechnology, USA)
13. Thermostatic Circulator (Multiphor III, Pharmacia, LKB Biotechnology, USA)
14. ELISA Plate reader (CERES UV900C, Biotex Instrument, USA)
15. Automated Coagulation Timer (Behring, Germany)
16. Transfer Electrophoresis Unit (Hoefer TE22, USA)
17. Transfer Unit (Pharmacia, Biotech Water, USA)
18. Water bath 60°C (Pharmacia, LKB Biotechnology, USA)

4.1.2. Supplies

1. Automatic pipettes (Socorex, Switzerland)
2. Autopipette tips (Corning Incorporated, Mexico)
3. Serological pipettes: 5 ml, 10 ml (HBG, Germany)
4. Pasteur pipettes (Costar®, USA)
5. Disposable microtiter plates (F96 Maxisorp Nunc-Immuno plate, USA)
6. Nitrocellulose membrane pore size 0.45 μm (Probind 45 membrane roll, Phamacia Biotech, USA)
7. Gel Bond PAC film (Code No. 80-1129-36, Phamacia Biotech, USA)
8. Two glass plates size 11x13x 0.3 cm.
9. U-shaped spacer 1.5 mm.
10. Clip clamp size 1.5 x 3 cm.
11. Sponge 6 mm.
12. Sponge 3 mm.
13. Blotting paper
14. Cassette for blotting
15. Plastic container size 12x12 cm.
16. Forceps
17. Clamp scissors
18. Application strip (LKB, Pharmacia, Sweden)
19. Electrode wicks , 125x 260 mm. (LKB 1850-912 Pharmacia, Sweden)
20. 50 ml. disposable syringe with plastic tube 15x0.4 cm.
21. Plastic transfer pipette
22. Boiling water bath
23. Test tube 13x100 mm.
24. 3.2% buffer citrate solution tubes (BD Vacutainer sterile 1.8 ml.,USA)

4.1.3. Reagents

1. Anti-human von Willebrand factor rabbit IgG (Dako Ltd. Code A082, USA)
2. Anti-human von Willebrand factor rabbit IgG conjugated peroxidase (Dako Ltd. Code P226, USA)
3. OPD substrate tablets, 2 mg. (Dako code S2045, USA)
4. 0.05 M carbonate buffer pH 9.6
5. 0.02 M PBS-Tween pH 7.2
6. 100 mM citrate phosphate buffer pH 5.0
7. 30% hydrogen peroxide
8. 1 M H_2SO_4
9. Tween 20 (Sigma, USA)
10. Commercial standard VWF:Ag (Dade Behring, Germany)
11. Control plasma N (Dade Behring ORKE, Germany)
12. Control plasma P (Dade Behring, Germany)
13. Tris (Tris hydroxymethyl aminomethane, Trizma Base,Sigma, USA)
14. Glycine (Sigma, USA)
15. SDS (Sodium Dodecly Sulfate Sodium Salt/Lauryl sulfate) (Sigma, USA)
16. Absolute methanol
17. Thiomesol (Sigma, USA)
18. PBS Dulbucca (Biochrom KG, USA)
19. Bromphenol blue
20. Seakem Agarose HGT (high gelling temperature, ultra-pure) (FMC, USA)
21. Antifoam A : Sigma A-5758, USA)
22. Non fat dry milk (Mission brand, Thailand)
23. Bovine Serum Albumin (Sigma, USA)
24. Streptavidin biotinylated HRP complex (RPN 1031 Amersham, USA)

25. Biotinylated goat Anti-Rabbit IgG (H&L) (RPN 480 Amersham, USA)
26. Anti-human VWF rabbit IgG (A0082 DAKO, USA)
27. Von Willebrand reagent (Dade Behring OUBD2, Germany)
28. Standard human for VW reagent (Dade Behring ORKL13, Germany)
29. Bovine thrombin (1000 units) (Diagnostic Reagent Ltd. Thame, Oxan, England)
30. Thromborel S (Dade Behring, Germany)
31. Actin FS (Dade Behring, Germany)

4.2 Methods

4.2.1 Subjects

Study in 101 patients who were suspected to have dengue virus infection and admitted at the Department of Pediatrics, Faculty of Medicine Ramathibodi Hospital in Bangkok between August 2002 and November 2003. Enrollment criteria were age 5 through 15 years. Fifty eight males and 43 females were included. The subjects consisted of 21 patients with DF, 30 patients with DHF grade I, 33 patients with DHF grade II and 17 patients with DHF grade III&IV (DSS). Controls were represented by 7 patients with other febrile illness (OFIs). OFIs were defined as those patients who had no dengue virus-specific IgM and IgG or HAI antibody responses. Subject's data were collected from medical records. Fever day 0 was the day of defervescence. Day prior to fever day 0 were designated as fever day -1 (1 day prior to defervescence), fever day -2 as 2 day prior to defervescence and so on. The days after defervescence were fever day +1 and +2 respectively. Ethical approval of the study was obtained from the Committee on Human Rights Related to Researches involving Human Subjects of Faculty of Medicine Ramathibodi Hospital, Mahidol University, Bangkok.

4.2.2 Samples collection

Blood samples for this study were collectd as EDTA blood for 3 ml, citrated blood for 1.8 ml and clotting blood for 1 ml on the first day of admission and there were collected daily until the patient was discharged from the hospital. Convalescent-phase blood samples were collected 2-3 weeks after discharged. The blood samples were studied for the assessment of hematological analysis, screening coagulogram, von Willebrand factor antigen, ristocetin cofactor activity and von Willebrand factor multimer. Plasma and serum were separated as quickly as possible and stored at -152°C. Clinical suspected dengue virus infections were confirmed serologically with samples obtained from acute and convalescent phase of illness by IgM and IgG ELISA or by hemagglutination inhibition (HAI) assay from the Center for Vaccine Development, Salaya campus. Standard criteria were used to identify acute primary and acute secondary dengue virus infection (80).

4.2.3 Hematological analysis

EDTA blood samples were analyzed within 4 hrs after collection using a Technicon H*3 hematology analyzer for hematocrit (Hct) and platelet count (Plt).

4.2.4 Coagulation screening Test (APTT, PT and TT)

Platelet poor plasma (PPP) are examined within 4 hr after collection using a semi-automated blood coagulation analyzer Sysmex CA50

4.2.5 Assay for von Willebrand factor antigen (VWF:Ag)

PPP are stored in freezer -152°C until testing by ELISA assay as following: A polystyrene microtiter plate was coated with rabbit anti-human VWF IgG 100 µl in each wells and left overnight in moist chamber at 4°C (anti-human VWF diluted to 1:2000 in carbonate buffer). The plate was washed 3 times with PBS-Tween. Plasma patients were diluted to 1:100 in PBS-Tween and 100 µl is applied to

the wells (plasma standard diluted in serial dilution from 1:50 to 1:6400, plasma control diluted to 1:100). After incubation for 2 hours at 37°C, the plate was washed 3 times with PBS-Tween and incubated with 100 µl anti VWF conjugated-peroxidase diluted 1:2000 in PBS-Tween for 1 $^{1}/_{2}$- 2 hours at 37°C. The plate was then washed 3 times with PBS-Tween and 100 µl OPD substrate added (OPD substrate was freshly prepared by using OPD 4 tablets and citrate phosphate 12 ml and 30% H_2O_2 20 µl, protected solution from light). The color development was allowed to proceed for 5-10 minutes at room temperature and reaction was stopped by adding 100 µl 1M H_2SO_4. The intensity of this colored is directly proportional to the concentration of VWF:Ag present in the sample. Absorbance 492 nm was read by using microplate reader. Standard samples with known concentrations of VWF:Ag, provided by the manufacturer, were used for the creation of the standard curve and subsequently for the determination of the concentration of VWF:Ag in each plasma sample obtained from the dengue and non-dengue patients (32-34).

4.2.6 Assay for Ristocetin cofactor activity (VWF:RcoF)

VWF:RcoF was measure on the Behring Coagulation Time (BCT) automated coagulation analyzer with commercial von Willebrand reagent kit (Dade Behring OUBD2, Germany). All tests were carried out on undiluted plasma according to the manufacturer's protocol. Principle of the method was described: The stabilized human platelets, together with ristocetin, will agglutinate in the presence of von Willebrand factor. These agglutinations cause an increase in turbidity which can then be measured by an instrument. Assay for von Willebrand factor multimer (VWF multimer)

4.2.7 Assay for von Willebrand factor multimer (VWF:multimer)

PPP are stored in freezer -152°C until testing by SDS-AgaroseGel Electrophoresis (SDS-AGE) using a sensitive peroxidase staining method. PPP proteins are partially denatured using heat and an anionic detergent, Sodium dodecyl sulphate (SDS). This detergent binds to all plasma proteins. The amount of anion

bound to the protein is directly proportional to the size of that protein. Thus, when SDS treated plasma protein are subjected to an electric current, they will migrate towards the positive electrode (anode). If force to move through a "sieving" medium, smaller proteins will migrate faster than larger proteins and a size dependent distribution of plasma proteins are the result. This method utilizes an electrophoresis apparatus to provide electric current, and agarose to provide sieving. The use of the continuous system provides easier working than the discontinuous system. After electrophoresis, gel was blotted with nitrocellulose in a Tran –Blot cell. VWF polymer are detected by anti-human VWF. And counter stain with Biotinylated anti-rabbit and Strepavidin biotinylated HRP. Final developing with DAB-substrate (35, 36).

4.2.8 Statistical Analysis

For analysis, disease severity was compared among four severity groups based on clinical assessment (DF, DHF I, DHF II and DSS). The non-parametric Mann-Whitney U test was used to calculate statistical significance for the differences in von Willebrand factor antigen (VWF:Ag), ristocetin cofactor activity (VWF:RcoF) and in other hematologic parameters between the groups of patients and control subjects. Comparisons between results obtained at different time points were performed by use of the Wilcoxon signed-rank test. Differences were considered significant when the p-value was less than 0.05.

CHAPTER 5
RESULTS

5.1 Subject characteristics

From August 2002 through November 2003, 101 patients who were suspected to have dengue virus infection admitted at the Department of Pediatrics, Faculty of Medicine, Ramathibodi Hospital were recruited to the study. The subset population included 73 subjects with DHF, 21 with DF and 7 with OFIs. Among the subjects with DHF, 30 (41.1%) were grade I, 33 (45.2%) were grade II and 10 (13.7%) were grade III&IV (DSS). There were 58 males and 43 females and the male to female ratio was 1.3:1. The distribution by clinical diagnosis and corresponding demographic information are shown in **Table 5.1**.

Table 5.1 Diagnosis and demographic information for enrolled patients.

Diagnosis	Number of patients	Age (years) Mean (range)	Sex Male	Sex Female
Dengue fever	21	10.0 (5-15)	12	9
DHF grade I	30	10.0 (4-16)	18	12
DHF grade II	33	11.0 (6-14)	21	12
DSS	10	10.0 (4-14)	4	6
OFIs	7	12.0 (6-15)	3	4
Total	101	11.0 (4-16)	58	43

5.2 Hematological analysis

EDTA blood samples from various types of dengue virus infection and OFIs were analyzed by a Technicon H*3 hematology analyzer for hematocrit (Hct) and platelet count (Plt).

5.2.1 Hematocrit (Hct)

The patients with dengue fever (DF) had mean rising of Hct less than 20% whereas the patients with DHF have mean rising Hct higher than 20% (20.1% in grade I, 21.1% in grade II and 29.3% in DSS) as compared with 8% in OFIs patients **(Table 5.2)**. The mean Hct in each grade of DHF patients was highest at day of defervescence and after that, the mean Hct began to decline. The mean Hct was highest in DSS patients **(Figure 5.1)**. Mean Hct were significantly higher in patients with all grades of DHF than DF on day 0 ($p < 0.01$) **(Table 5.3)**.

Table 5.2 Mean of maximum and minimum Hct in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grades I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients.

Type	DF	DHF I	DHF II	DSS	OFIs
Number of patients	21	30	33	10	7
Max Hct (%)	38.6 ± 6.1	42.9 ± 3.9	43.6 ± 4.2	46.3 ± 3.9	37.9 ± 7.5
Min Hct (%)	36.4 ± 6.2	35.7 ± 3.5	36.0 ± 4.1	35.8 ± 2.6	35.1 ± 6.5
Mean increment in Hct (%)	6.0	20.1	21.1	29.3	8.0

Table 5.3 Mean ± SD of hematocrit in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness.

Hematocrit (%)							
Type	Day -3	Day -2	Day -1	Day 0	Day 1	Day 2	Conval*
DF (n)	35.4 ± 2.0 (3)	35.5 ± 2.5 (6)	36.6 ± 3.5 (14)	36.1 ± 3.0 # (18)	37.6 ± 7.6 (14)	40.3 ± 11.7 (5)	36.2 ± 2.1 (9)
DHF I (n)	36.2 ± 1.1 (2)	37.9 ± 3.3 (6)	38.1 ± 3.2 (18)	39.1 ± 2.9 (26)	38.5 ± 3.9 (24)	39.7 ± 3.5 (8)	35.4 ± 2.9 (22)
DHF II (n)	41.1 ± 5.0 (3)	40.1 ± 3.5 (8)	38.1 ± 4.4 (19)	39.6 ± 4.1 (26)	38.7 ± 4.4 (26)	35.9 ± 4.1 (13)	35.6 ± 2.7 (22)
DSS (n)	38.7 (1)	41.5 ± 3.2 (3)	41.3 ± 2.6 (2)	41.8 ± 3.7 (6)	41.3 ± 5.6 (9)	35.8 ± 2.6 (7)	33.1 ± 1.3 (4)
OFIs (n)	33.0 (1)	35.7 ± 5.3 (2)	39.9 ± 15.1 (2)	37.9± 8.2 (6)	38.6 ± 7.4 (4)	38.6 (1)	34.3 ± 0.2 (2)

Conval* = Convalescence;

DF vs. DHF I, DHF II and DSS: # $p < 0.05$;

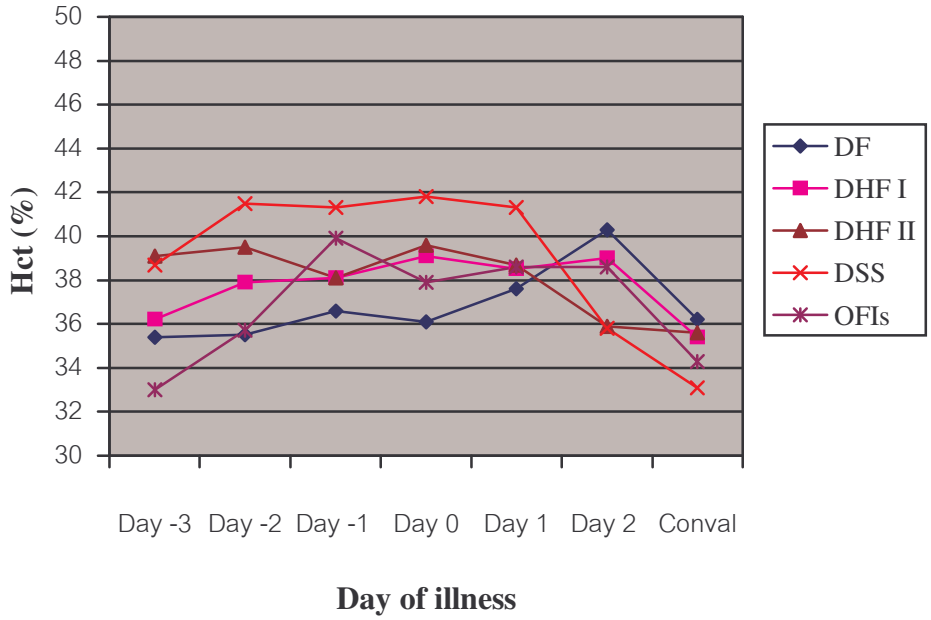


Figure 5.1 Mean of hematocrit in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grades I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness.

5.2.1 Platelet counts (Plt)

Both DF and DHF patients had mean platelet number drops below 100,000 cells/ μL for 1-2 day before shock/defervescence and began to rise on day 1 for DF patients whereas the other DHF patients began to rise on day 2 **(Figure 5.2)**. One day before defervescence (day –1), the mean platelet in DF patients was significantly higher than DHF II and DSS ($p < 0.05$), but lower than OFIs patients ($p < 0.05$). On day 0, the mean platelet in DF patients was significantly higher than DHF/DSS ($p < 0.05$), but lower than OFIs patients ($p < 0.05$). On day 0 the mean platelet in patients with DSS was significantly lower than in DF, DHF I, DHF II and OFIs patients ($p < 0.05$). On day 1, the mean platelet in DHF/DSS patients was significantly lower than in OFIs and DF patients ($p < 0.01$) and the mean platelet in DSS patients was significantly lower than DF, DHF I and DHF II patients ($p < 0.05$). On day 2, the mean platelet in DF patients was significantly higher than DHF/DSS ($p < 0.05$) **(Table 5.4)**. Within group of patients, the mean platelet in DHF I groups were significantly higher on day -1 than day 0 ($p < 0.01$) and day -2 than day -1 ($p < 0.05$). The mean platelets in DHF II and DSS groups were significantly higher on day of convalescence than day 2 ($p < 0.05$). The mean platelet in DSS groups were significantly higher on day 2 than day 1 ($p < 0.05$) **(Table 5.5)**.

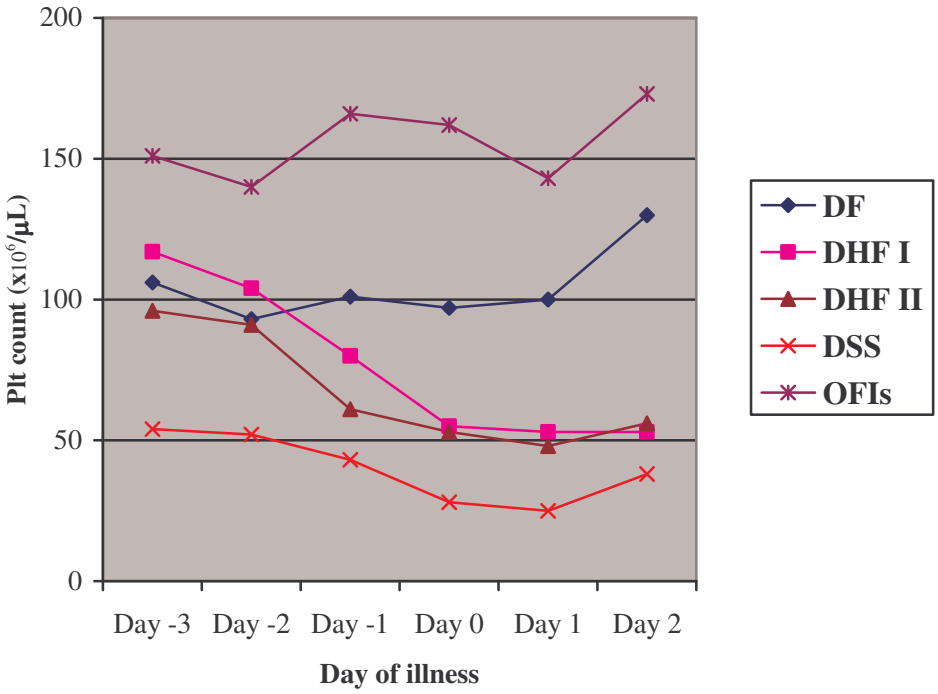


Figure 5.2 Mean number of platelet counts in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grades I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness.

Table 5.4 Mean ± SD of platelet counts in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grades I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness.

	Platelet count ($x10^6/\mu L$)						
Type	Day -3	Day -2	Day -1	Day 0	Day 1	Day 2	Conval*
DF (n)	106± 39 (3)	93 ± 21 (6)	101 ± 27 ‡ (14)	97 ± 30 ‡‡ (18)	100± 30 ‡‡ (14)	130 ± 46‡‡ (5)	391 ± 121 (9)
DHF I (n)	117± 96 (2)	104 ± 38 (6)	80 ± 32 (18)	55 ± 27 (26)	53 ± 23 (24)	53 ± 27 (8)	378 ± 159 (22)
DHF II (n)	96 ± 44 (3)	91 ± 27 (8)	61 ± 33 (19)	53 ± 27 (26)	48 ± 26 (26)	56 ± 37 (13)	374 ± 107 (22)
DSS (n)	54 (1)	52 ± 13 (3)	43 ± 8 (2)	28 ± 22 ¥ (6)	25 ± 13 ¥ (9)	38 ± 15 (7)	397 ± 105 (4)
OFIs (n)	151 (1)	140 ± 20 (2)	166 ± 62** (2)	162 ± 58** (6)	143 ± 57¶ (4)	173 (1)	150 ± 100 (2)

Conval* = Convalescence.

OFIs vs. dengue (DF+DHF): ** $p < 0.05$ / OFIs vs. DHF: ¶ $p < 0.01$;

DF vs. DHF II and DSS: ‡ $p < 0.05$ / DF vs. DHF: ‡‡ $p < 0.05$;

DSS vs. DF, DHF I and DHF II: ¥ $p < 0.05$

Table 5.5 Comparison of platelet counts on day -2, -1, 0, 1, 2 and day of convalescence in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grades I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients.

	p - value*				
Platelet	Day -2 vs. day -1	Day -1 vs. day 0	Day 0 vs. day 1	Day 1 vs. day 2	Day 2 vs. Conval
DF	NS	NS	NS	NS	NS
DHF I	0.03	< 0.01	NS	NS	NS
DHF II	NS	NS	NS	NS	0.01
DSS	-	NS	NS	0.04	0.02
OFIs	-	NS	NS	-	-

* $p < 0.05$ for the differences within groups (Wilcoxon signed-rank test); NS = not significant

5.3 Coagulation screening tests

Citrated plasma samples from various types of dengue virus infection and OFIs were analyzed by a semi-automated blood coagulation analyzer Sysmex CA50 for activated partial thromboplastin time (APTT), prothrombin time (PT) and thrombin time (TT).

5.3.1 Activated partial thromboplastin time (APTT)

The value of APTT in patients with DF, DHF I, DHF II and OFIs did not show the significant difference among them in all days of illness and convalescence day, but in DSS patients, the value of APTT was significantly higher than other groups of patients on day -1, day 0 and day 1 ($p< 0.05$), except on day -1, the value of APTT did not show the significant difference among DSS and DHF I patient as shown in **Figure 5.3** and **Table 5.6**. Within group of patients, the values of APTT in DF group were significantly higher on day -1, day 0 and day 1 than convalescence day ($p< 0.05$). In DHF I group, the value of APTT was significantly higher on day 0 than day 1 and day 1 than day 2 ($p< 0.05$) as on day -1, day 0 and day 1, the value of APTT was significantly higher than day of convalescence ($p< 0.05$). Within group of DHF II, the value of APTT was significantly higher on day -1, day 0, day 1 and day 2 than day of convalescence ($p< 0.01$). The value of APTT in DSS was not significant difference among all days of illness **(Table 5.7)**. However, the mean APTT values of DHF II and DSS group was slightly higher than the normal range.

Table 5.6 Mean ± SD of activated partial thromboplastin time (APTT) in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness

APTT (seconds)							
Type	Day -3	Day -2	Day -1	Day 0	Day 1	Day 2	Conval*
DF (n)	37.3 ± 4.2 (3)	38.5 ± 4.9 (6)	36.9 ± 3.7 (14)	36.6 ± 4.1 (18)	36.3 ± 3.5 (15)	37.1 ± 6.2 (4)	30.9 ± 1.9 (9)
DHF I (n)	36.7 ± 8.9 (2)	35.6 ± 3.8 (6)	40.4 ± 7.5 (18)	39.3 ± 5.3 (26)	38.8 ± 5.3 (24)	37.0 ± 5.0 (8)	30.2 ± 2.0 (22)
DHF II (n)	39.9 ± 2.6 (3)	39.5 ± 5.6 (8)	40.0 ± 6.6 (19)	39.9 ± 5.2 (26)	40.1 ± 6.4 (26)	39.5 ± 5.0 (13)	30.3 ± 1.8 (22)
DSS (n)	38.9 ± 0.0 (1)	41.6 ± 6.5 (3)	44.7± 4.2 ¶ (9)	45.3± 4.4 # (9)	43.3± 3.7 # (9)	40.1 ± 3.0 (6)	30.6 ± 2.8 (4)
OFI (n)	30.2 ± 0.0 (1)	37.0 ± 2.8 (2)	38.8 ± 7.5 (2)	35.4 ± 5.0 (6)	34.6 ± 6.4 (4)	36.2 ± 0.0 (1)	31.9 ± 0.1 (2)

Conval* = Convalescence;

DSS vs. DF, DHF II and OFIs: ¶ $p < 0.05$;

DSS vs. all groups: # $p < 0.05$

Table 5.7 Comparison value of activated partial thromboplastin time (APTT) on day -2, -1, 0, 1, 2 and convalescence day in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II and dengue shock syndrome (DSS).

APTT	p-value*								
	Day -2 vs. Day -1	Day -1 vs. Day 0	Day 0 vs. Day 1	Day 0 vs. Day 2	Day 1 vs. Day 2	Day -1 vs. Conval	Day 0 vs. Conval	Day 1 vs. Conval	Day 2 vs. Conval
DF	NS	NS	NS	NS	NS	0.004	0.002	0.017	NS
DHF I	NS	NS	0.032	NS	0.021	0.012	0.005	0.003	NS
DHF II	NS	NS	NS	NS	NS	0.000	0.000	0.000	0.008
DSS	NS	NS	NS	NS	NS	NS	NS	NS	NS

* $p < 0.05$ for the differences within groups (Wilcoxon signed-rank test); NS = not significant

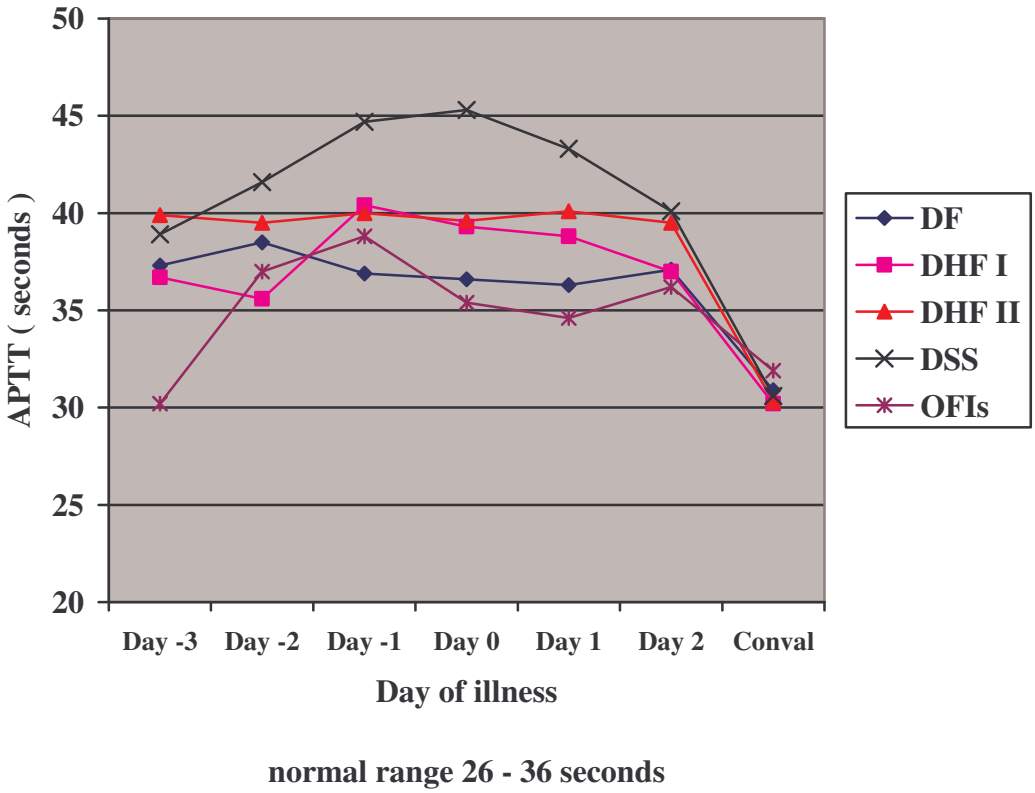


Figure 5.3 Mean value of activated partial thromboplastin time (APTT) in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness.

5.3.2 Prothrombin time (PT)

The value of PT in patients with DF, DHF I, DHF II and OFIs did not show the significant difference among them in all days of illness and convalescence day, but in DSS patients, the value of PT was significantly higher than other groups of patients on day -1, day 0 and day 1 ($p< 0.05$), except on day -1, the value of PT did not show the significant difference among DSS and DHF I patient as shown in **Figure 5.4** and **Table 5.8**. Within group of patients, the values of PT in DF group were significantly higher on day -1, day 0 and day 1 than convalescence day ($p< 0.05$). In DHF I group, the value of PT was significantly higher on day 0 than day 1 and day 1 than day 2 ($p< 0.05$) as on day -1, day 0 and day 1, the value of PT was significantly higher than day of convalescence ($p< 0.05$). Within group of DHF II, the value of PT was significantly higher on day -1, day 0, day 1 and day 2 than day of convalescence ($p< 0.01$). The value of PT in DSS was not significant difference among all days of illness **(Table 5.9)**.

Table 5.8 Mean ± SD of prothrombin time (PT) in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness

PT (seconds)							
Type	Day -3	Day -2	Day -1	Day 0	Day 1	Day 2	Conval*
DF (n)	12.9 ± 0.5 (3)	13.7 ± 1.2 (6)	12.8 ± 0.9 (14)	12.6 ± 0.8 (18)	12.3 ± 0.9 (15)	12.0 ± 1.4 (4)	11.6 ± 0.8 (9)
DHF I (n)	12.6 ± 1.6 (2)	12.4 ± 1.0 (6)	14.6 ± 5.9 (18)	12.7 ± 1.7 (26)	12.5 ± 1.4 (24)	11.9 ± 1.7 (8)	11.5 ± 1.0 (22)
DHF II (n)	13.7 ± 0.6 (3)	12.9 ± 0.4 (8)	13.5 ± 2.5 (19)	12.6 ± 1.1 (26)	12.3 ± 1.9 (26)	12.3 ± 1.8 (13)	11.4 ± 0.9 (22)
DSS (n)	12.4 ± 0.0 (1)	13.9 ± 2.2 (3)	16.0 ± 4.0 (9)	15.2 ± 2.8 # (9)	14.4 ± 2.1 # (9)	11.7 ± 0.9 (6)	12.4 ± 0.4 (4)
OFI (n)	16.1 ± 0.0 (1)	13.3 ± 1.1 (2)	15.0 ± 2.3 (2)	13.0 ± 1.8 (6)	12.7 ±1.8 (4)	12.5 ± 0.0 (1)	12.3 ± 0.4 (2)

Conval* = Convalescence;

DSS vs. DF, DHF I and DHF II: # $p < 0.05$

Table 5.9 Comparison value of prothrombin time (PT) on day -2, -1, 0, 1, 2 and convalescence day in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II and dengue shock syndrome (DSS).

PT	p-value*								
	Day -2 vs. Day -1	Day -1 vs. Day 0	Day 0 vs. Day 1	Day 0 vs. Day 2	Day 1 vs. Day 2	Day -1 vs. Conval	Day 0 vs. Conval	Day 1 vs. Conval	Day 2 vs. Conval
DF	NS	NS	0.004	NS	NS	0.008	0.003	NS	NS
DHF I	NS	NS	NS	0.042	0.017	0.018	0.012	0.003	NS
DHF II	NS	0.019	0.012	NS	NS	0.002	0.001	NS	NS
DSS	NS	0.020	NS	0.018	0.034	NS	NS	NS	NS

* $p < 0.05$ for the differences within groups (Wilcoxon signed-rank test); NS = not significant

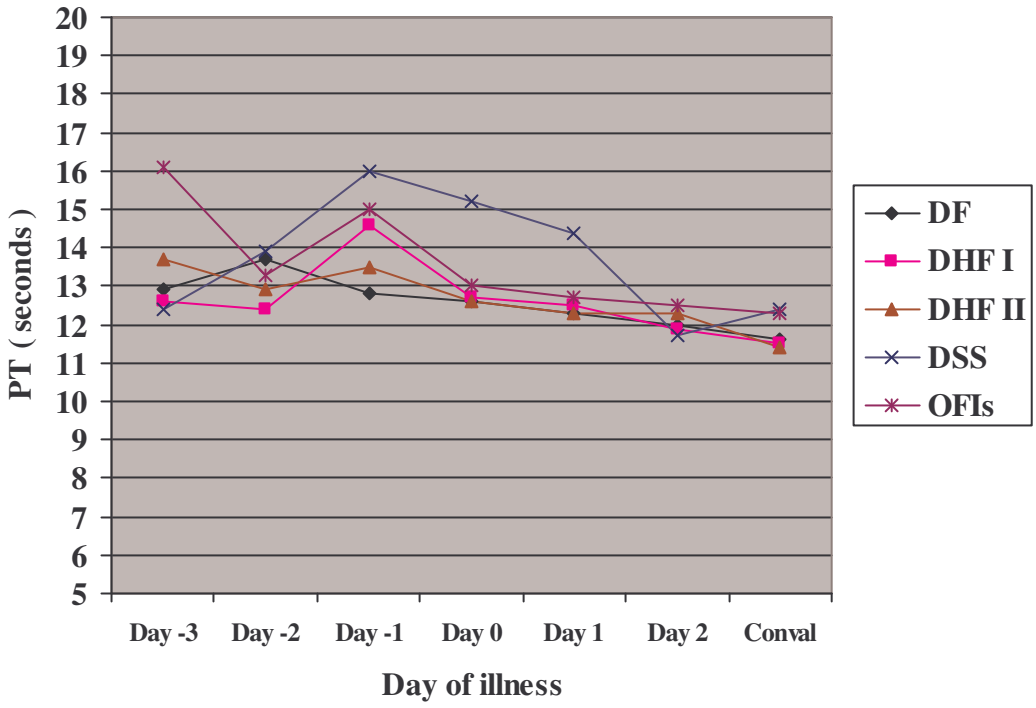


Figure 5.4 Mean value of prothrombin time (PT) in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness.

5.3.3 Thrombin time (TT)

The value of TT in patients with DF, DHF I, DHF II and OFIs did not show the significant difference among them in all days of illness and convalescence day, but in DSS patients, the value of PT was significantly higher than other groups of patients on day -2, day -1 and day 1 ($p< 0.05$), except on day -1, the value of TT did not show the significant difference among DSS and DHF I patient as shown in **Figure 5.5** and **Table 5.10** Within group of patients, the value of TT in DF group were significantly higher on day -1, day 0 and day 1 than convalescence day ($p< 0.05$). In DHF I group, the value of TT was significantly higher on day 1 than day 2 and day 0 than day 2 ($p< 0.05$) as on day -2 day 0 and day 1, the value of TT was significantly higher than day of convalescence ($p< 0.05$). Within group of DHF II, the value of TT was significantly higher on day -1, day 0, day 1 and day 2 than day of convalescence ($p< 0.01$). The value of TT in DSS was not significant difference among all days of illness **(Table 5.11)**.

Table 5.10 Mean ± SD of thrombin time (TT) in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness

TT (seconds)							
Type	Day -3	Day -2	Day -1	Day 0	Day 1	Day 2	Conval*
DF (n)	12.2 ± 0.3 (3)	13.4 ± 1.9 (6)	13.9 ± 1.8 (14)	13.5 ± 1.5 (18)	13.7 ± 1.8 (15)	14.3 ± 1.8 (4)	11.5 ± 0.5 (9)
DHF I (n)	16.1 ± 6.0 (2)	13.0 ± 1.9 (6)	14.3 ± 2.4 (18)	14.1 ± 1.6 (26)	14.4 ± 2.1 (24)	14.7 ± 2.5 (8)	11.7 ± 0.7 (22)
DHF II (n)	14.8 ± 1.2 (3)	12.4 ± 3.0 (8)	14.2 ± 3.2 (19)	14.9 ± 2.4 (26)	15.0 ± 2.4 (26)	13.9 ± 1.8 (13)	11.5 ± 0.9 (22)
DSS (n)	15.0 ± 0.0 (1)	17.9 ± 5.9 (3)	16.0 ± 1.1 ¶ (9)	16.5 ± 3.1 # (9)	16.8 ± 3.8 # (9)	14.9 ± 1.4 (6)	11.3 ± 0.5 (4)
OFI (n)	14.5 ± 0.0 (1)	13.5 ± 0.8 (2)	13.0 ± 2.1 (2)	12.6 ± 0.5 ¥ (6)	12.8 ±1.0 § (4)	13.0 ± 0.0 (1)	11.2 ± 0.4 (2)

Conval* = Convalescence;

DSS vs. all groups: ¶ $p < 0.05$;

DSS vs. DF, DHF I and OFIs: # $p < 0.05$;

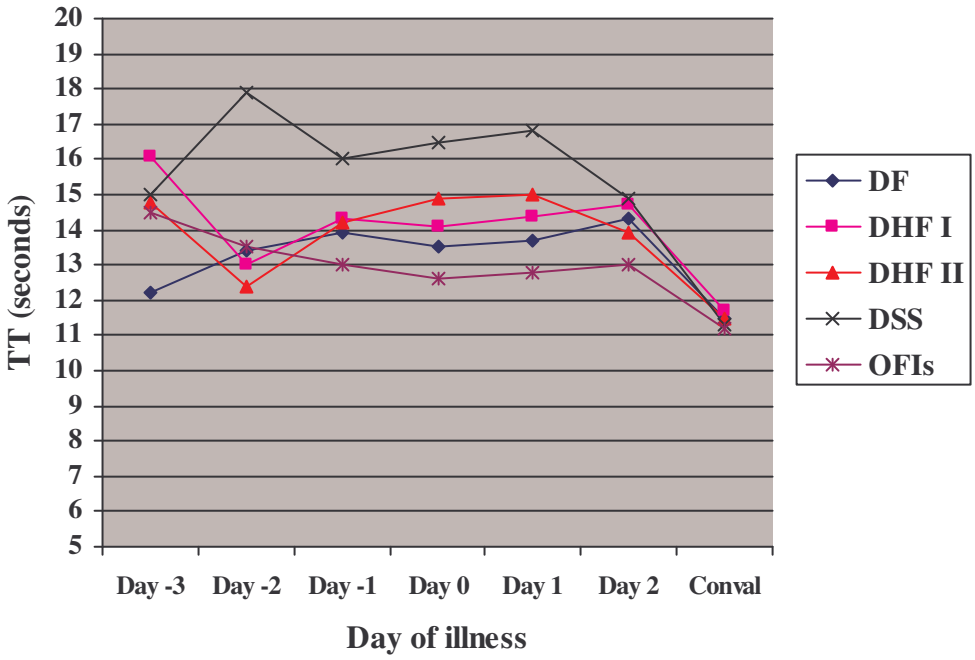
OFIs vs. all groups: ¥ $p < 0.05$;

OFIs vs. DHF I, DHF II and DSS: § $p < 0.05$

Table 5.11 Comparison value of thrombin time (TT) on day -2, -1, 0, 1, 2 and convalescence day in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II and dengue shock syndrome (DSS).

TT	p-value*								
	Day -2 vs. Day -1	Day -1 vs. Day 0	Day 0 vs. Day 1	Day 0 vs. Day 2	Day 1 vs. Day 2	Day -1 vs. Conval	Day 0 vs. Conval	Day 1 vs. Conval	Day 2 vs. Conval
DF	NS	NS	NS	NS	NS	0.008	0.001	0.011	NS
DHF I	0.035	NS	NS	NS	NS	0.012	0.005	0.010	NS
DHF II	NS	NS	NS	NS	0.045	0.001	0.000	0.000	0.025
DSS	NS	NS	NS	NS	NS	NS	NS	NS	NS

* $p < 0.05$ for the differences within groups (Wilcoxon signed-rank test); NS = not significant



normal range 10 -13 seconds

Figure 5.5 Mean value of thrombin time (TT) in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness.

5.4 Von Willebrand Factor antigen (VWF:Ag)

The value of VWF:Ag in patients with DF, DHF I, DHF II and OFIs did not show the significant difference among them in all days of illness and convalescence day, but in DSS patients, the value of VWF:Ag was significantly higher than other groups of patients on day -1, day 0, day 1 and day 2 ($p< 0.05$), except on day 2, the value of VWF:Ag did not show the significant difference among DSS and OFIs patient as shown in **Figure 5.6** and **Table 5.12**. Within group of patients, the value of VWF:Ag in DF group were significantly higher on day -1, day 0, day 1 and day 2 than convalescence day ($p< 0.05$). In DHF I group, the value of VWF:Ag was significantly higher on day 2 than day 0 and day 2 than day 1 ($p< 0.05$) as on day -1, day 0 and day 1, and day 2, the value of VWF:Ag was significantly higher than day of convalescence ($p< 0.05$). Within group of DHF II, the value of VWF:Ag was significantly higher on day 0 than day -1 ($p< 0.05$) as on day -1, day 0, day 1 and day 2, the value of VWF:Ag was significantly higher than day of convalescence ($p< 0.01$). The value of VWF:Ag in DSS was significantly higher on day 0 than day -1 ($p< 0.01$) as on day -1, day 0, day 1 and day 2, the value of VWF:Ag was significantly higher than day of convalescence ($p< 0.01$) **(Table 5.13)**.

Table 5.12 Mean ± SD of von Willebrand factor antigen (VWF:Ag) in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness

VWF:Ag (%)							
Type	Day -3	Day -2	Day -1	Day 0	Day 1	Day 2	Conval*
DF (n)	198.0±11.8 (3)	202.0±6.3 (6)	200.0±5.0 (14)	199.3±7.9 (18)	202.8±8.2 (15)	199.8±10.1 (4)	114.7±24.9 (14)
DHF I (n)	204.3±13.4 (3)	196.4±12.3 (7)	202.5±10.1 (20)	206.3±11.9 (29)	205.1±18.5 (28)	213.6±13.7 (11)	108.2±25.0 (14)
DHF II (n)	203.3±15.1 (3)	192.5±9.6 (8)	201.0±13.0 (19)	210.2±16.6 (28)	209.9±18.3 (26)	212.3±14.2 (12)	107.2±21.5 (21)
DSS (n)	220.0±0.0 (1)	212.7±1.7 (3)	217.0±14.6 # (9)	225.9±12.1 # (10)	230.2±10.0 # (9)	230.2±10.6 # (6)	108.3±31.2 (4)
OFIs (n)	195.0±0.0 (1)	206.0±7.1 (2)	197.0±3.8 ¥ (6)	200.3±9.3 ¥ (7)	204.2±7.2 ¥ (5)	190.0±0.0 (1)	112.0±11.3 (2)

Conval* = Convalescence;

DSS vs. DF, DHF I and DHF II: # $p < 0.05$,

OFIs vs. DSS: ¥ $p < 0.05$

Table 5.13 Comparison level of von Willebrand factor antigen (VWF:Ag) on day -2, -1, 0, day 1, day 2 and convalescence day in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II and dengue shock syndrome (DSS).

VWF:Ag	p-value*								
	Day -2 vs. Day -1	Day -1 vs. Day 0	Day 0 vs. Day 1	Day 0 vs. Day 2	Day 1 vs. Day 2	Day -1 vs. Conval	Day 0 vs. Conval	Day 1 vs. Conval	Day 2 vs. Conval
DF	NS	NS	NS	NS	NS	0.002	0.001	0.008	0.005
DHF I	NS	NS	NS	0.028	0.049	0.012	0.005	0.003	0.001
DHF II	NS	0.014	NS	NS	NS	0.000	0.000	0.000	0.008
DSS	NS	0.007	NS	NS	NS	0.000	0.000	0.000	0.000

* $p < 0.05$ for the differences within groups (Wilcoxon signed-rank test); NS = not significant

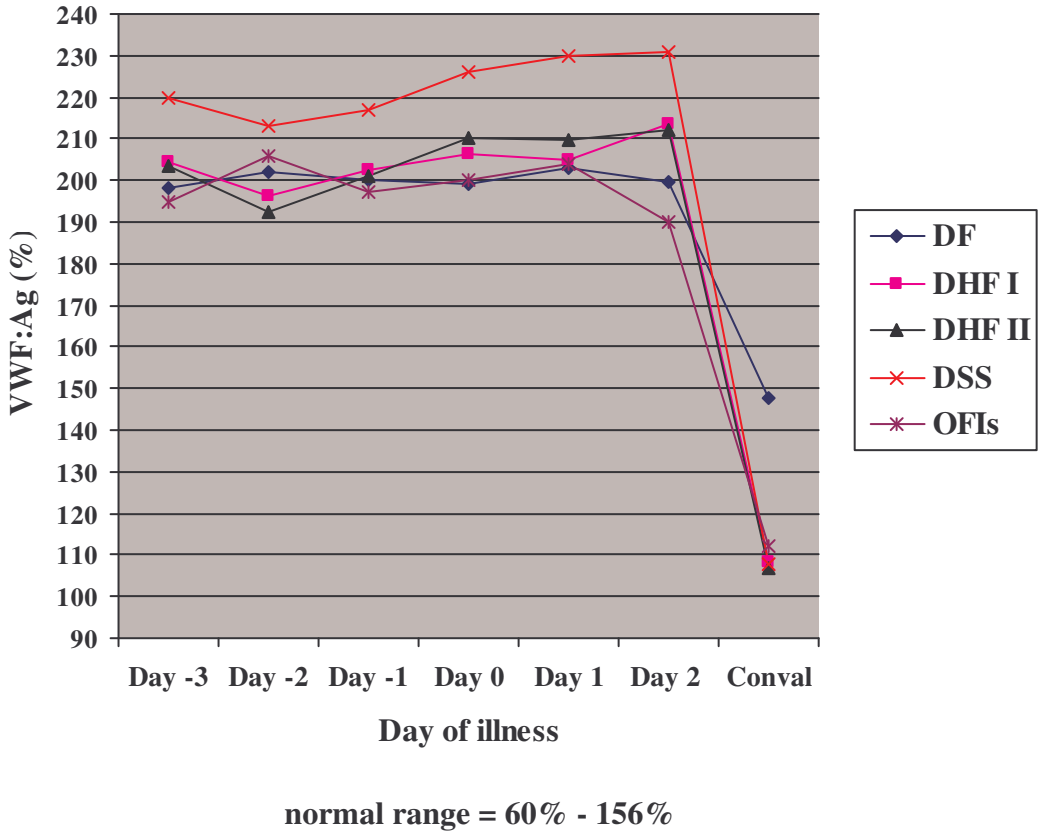


Figure 5.6 Mean level of von Willebrand factor antigen (VWF:Ag) in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness.

5.5 Ristocetin cofactor activity (VWF:RcoF)

The value of VWF:RcoF in patients with DF and OFIs did not show the significant difference among them in all days of illness and convalescence day, but in DHF I, DHF II and DSS patients, the value of VWF:RcoF was significantly higher than DF and OFIs patients on day -1, day 0, day 1 ($p< 0.05$), except on convalescence day. In DSS patients, the value of VWF:RcoF was significantly higher than other groups of patients on day -1, day 0, day 1 ($p< 0.05$), except on convalescence day, the value of VWF:RcoF did not show the significant difference among them as shown in **Figure 5.7** and **Table 5.14**. Within group of patients, the value of VWF:RcoF in DF, DHF I, DHF II and DSS group were significantly higher on day -1, day 0, day 1 than convalescence day ($p< 0.05$). In DSS group, the value of VWF:RcoF was significantly higher on day 0 than day -1($p< 0.05$). The value of VWF:RcoF in patients with DF, DHF I and DHF II did not show the significant difference among them on day -1 with day 0 and day 0 with day 1 ($p < 0.05$) **(Table 5.13)**.

Table 5.14 Mean ± SD of the ristocetin cofactor activity (VWF:RcoF) in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness.

VWF:RcoF (%)				
Type	Day -1	Day 0	Day 1	Conval*
DF (n)	187.2 ± 9.9 # (14)	190.7 ± 11.2 # (19)	193.2 ± 17.0 # (16)	113.3 ± 18.1 (14)
DHF I (n)	201.0 ± 16.2 (21)	206.7 ± 13.7 (29)	208.3 ± 14.7 (29)	124.5 ± 18.0 (14)
DHF II (n)	203.0 ± 20.7 (19)	206.1 ± 18.7 (28)	208.3 ± 20.0 (27)	114.3 ± 15.8 (21)
DSS (n)	212.5 ± 10.9 (9)	226.4 ± 9.5 ¥ (10)	231.3 ± 9.7 ¥ (9)	136.75 ± 3.5 (4)
OFIs (n)	181.1 ± 11.6 § (6)	189.7 ± 17.3 § (7)	186.2 ± 22.4 § (5)	116.5 ± 2.12 (2)

Conval* = Convalescence;
DF vs. DHF I, DHF II and DSS: # $p < 0.05$,
DSS vs. DHF I, DHF II: ¥ $p < 0.05$,
OFIs vs. DHI I, DHF II and DSS: § $p < 0.05$

Table 5.15 Comparison level of ristocetin cofactor activity (VWF:RcoF) on day-1, 0, day 1 and convalescence day in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II and dengue shock syndrome (DSS).

VWF:RcoF	p-value*				
	Day -1 vs. day 0	Day -1 vs. Conval	Day 0 vs. day 1	Day 0 vs. Conval	Day 1 vs. Conval
DF	NS	0.002	NS	0.01	0.005
DHF I	NS	0.012	NS	0.005	0.003
DHF II	NS	0.000	NS	0.000	0.000
DSS	0.011	0.004	NS	0.003	0.000

* $p < 0.05$ for the differences within groups (Wilcoxon signed-rank test); NS = not significant

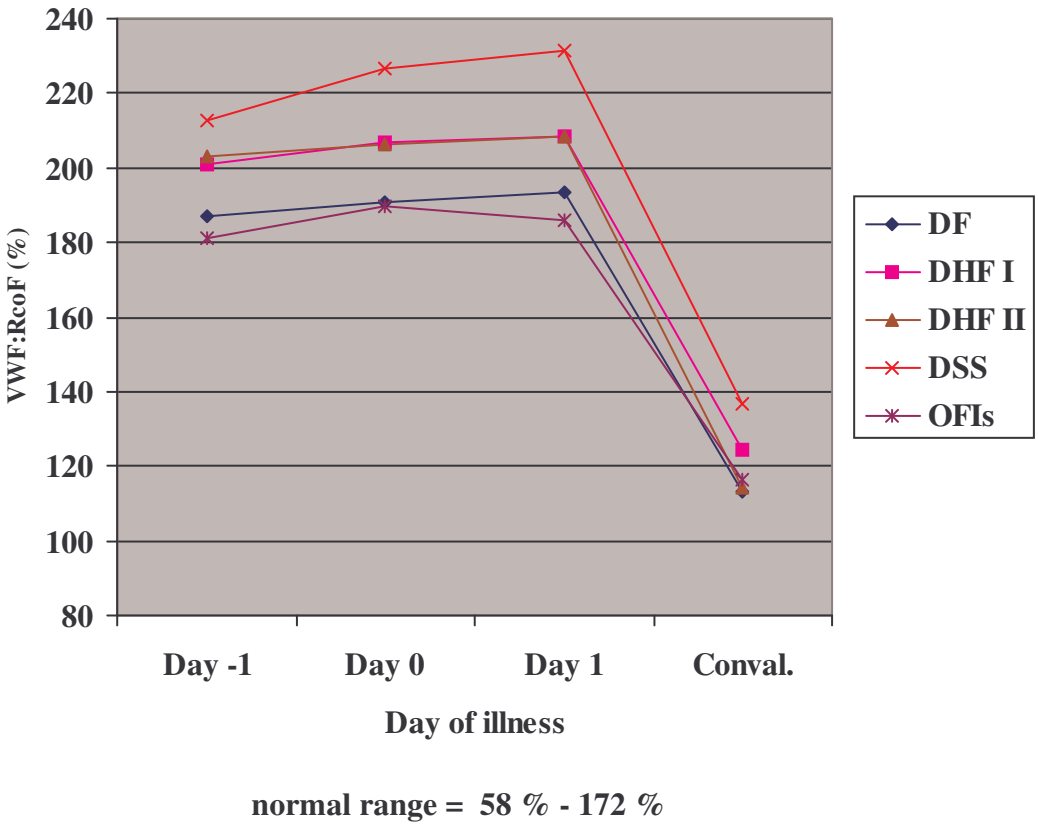
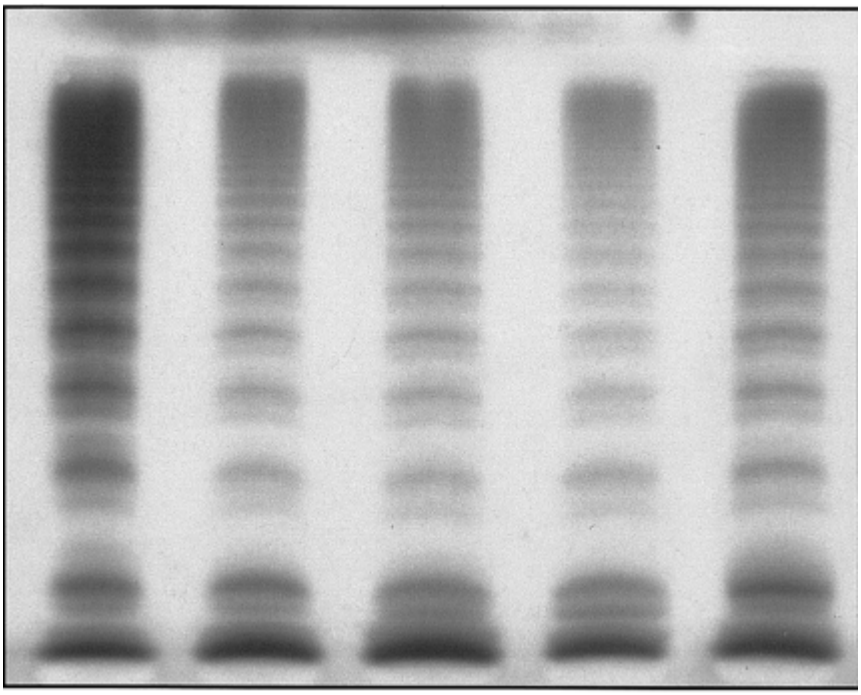


Figure 5.7 Mean level of ristocetin cofactor activity (VWF:RcoF) in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS) and other febrile illness (OFIs) patients, followed by the day of illness.

5.6 Von Willebrand Factor Multimer (VWF multimer)

In patients with DF, DHF I, DHF II and DSS did not show the abnormal band, ultra large VWF (ULVWF) multimers or the loss of high molecular weight (HMW) VWF mutimer on SDS-AgaroseGel Electrophoresis (SDS-AGE) using a sensitive peroxidase staining method, compared with normal plasma as shown in **Figure 5.8.**



Normal **DF** **DHF I** **DHF II** **DSS**

Figure 5.8 The sensitive peroxidase staining method of plasma VWF multimer pattern obtained by high-resolution agarose gel (2.5%) in patients with dengue fever (DF), dengue hemorrhagic fever (DHF) grade I, II, dengue shock syndrome (DSS), compared with normal plasma sample.

5.7 Prediction of dengue shock syndrome (DSS) by laboratory results

The assessments of the association between laboratory results (on day -1, day o and day 1) and the severity of the diseases have been showed with the relative risk as shown in **Table 5.16**. In patients with rising Hct ≥ 25% being at higher risk of contracting DSS was 7.7 (95% CI: 2.2-26.5, p< 0.05) and 11.5 (95% CI: 3.3-40.1, p< 0.05) of DHF patients and all patients.

The relative risk of patients with platelet count below 40,000 cells/µl being at higher risk of contracting DSS was 4.8 (95% CI: 1.1-21.3, p< 0.05) and 8.2 (95% CI: 1.9-36.7, p< 0.05) of DHF patients and all patients.

The relative risk of patients with rising Hct ≥ 25% and platelet count below 40,000 cells/µl was 6.9 (95% CI: 2.3-21.1, p< 0.05) and 10.2 (95% CI: 3.3-31.2, p< 0.05) of DHF patients and all patients.

The relative risk of patients with APTT ≥ 44 seconds being at higher risk of contracting DSS was 4.7 (95% CI: 1.3-16.8, p< 0.05) and 10.1(95% CI: 3.3-31.2, p< 0.05) of DHF patients and all patients.

The relative risk of patients with PT ≥ 14 seconds being at higher risk of contracting DSS was 7.1 (95% CI: 2.1-24.7, p< 0.05) and 8.9 (95% CI: 2.5-31.4, p< 0.05) of DHF patients and all patients.

The relative risk of patients with TT ≥ 16 seconds being at higher risk of contracting DSS was 9.9 (95% CI: 2.3-42.8, p< 0.05) and 15.3 (95% CI: 5.3-44.4, p< 0.05) of DHF patients and all patients.

The relative risk of patients with all prolonged screening coagulation test (APTT ≥ 44 seconds, PT ≥ 14 seconds and TT ≥ 16 seconds) being at higher risk of contracting DSS was 10.7 (95% CI: 3.7-30.6, p< 0.05) and 15.3 (95% CI: 5.3-44.4, p< 0.05) of DHF patients and all patients.

The relative risk of patients with VWF:Ag ≥ 210 % being at higher risk of contracting DSS was 10.1 (95% CI: 5.3-44.4, p< 0.05) and 10.9 (95% CI: 1.5-81.7, p< 0.05) of DHF patients and all patients. The relative risk of patients with VWF:RcoF ≥ 210 % being at higher risk of contracting DSS was 7.0 (95% CI: 0.93-52.6, p< 0.05) and 12.6 (95% CI: 1.7-96.0, p< 0.05) of DHF patients and all patients.

The relative risk of patients with VWF:Ag and VWF:RcoF ≥ 210 % being at higher risk of contracting DSS was 16.3 (95% CI: 2.2-121.4, p< 0.05) and 24.7 (95% CI: 3.3-185.7, p< 0.05)

Table 5.16 Comparison of laboratory findings in dengue shock syndrome (DSS) patients.

Variables	DSS positive/ DHF (%)	DSS positive/ all patients (%)	DSS in DHF RR	95% CI	DSS in all patients RR	95% CI
Laboratory findings						
Hematology						
Rising Hct ≥ 25%	7/73 (0.09)	7/101 (0.07)	7.7	2.2-26.5	11.5	3.3-40.1
Plt. Count ≤ 40,000	8/73 (0.11)	8/101 (0.08)	4.8	1.1-21.3	8.2	1.9-36.7
Rising Hct ≥ 25% and Plt. Count ≤ 40,000 cells/µl	6/73 (0.08)	6/101 (0.06)	6.9	2.3-21.1	10.2	3.3-31.2
Coagulation						
APTT ≥ 44 seconds	7/73 (0.09)	7/101 (0.07)	4.7	1.3-16.8	6.7	1.9-24.1
PT ≥ 14 seconds	7/73 (0.09)	7/101 (0.07)	7.1	2.1-24.7	8.9	2.5-31.5
TT ≥ 16 seconds	8/73 (0.11)	8/101 (0.08)	9.9	2.3-42.8	13.6	3.1-59.5
All prolonged coag.	6/73 (0.08)	6/101 (0.06)	10.7	3.7-30.6	15.3	5.3-44.4
Von Willebrand Factor						
VWF:Ag ≥ 210 %	9/73 (0.12)	9/101 (0.09)	10.9	1.5-81.7	15.6	2.1-118.1
VWF:RcoF ≥ 210 %	9/73 (0.12)	9/101 (0.09)	7.0	0.9-52.6*	12.6	1.7- 96.0
VWF:Ag and VWF:RcoF ≥ 210 %	9/73 (0.12)	9/101 (0.09)	16.3	2.2-121.4	24.7	3.3-185.7

RR = relative risk; CI = confidence interval;

DHF = DHF I, DHF II and DSS; DSS = DHF III and DHF IV;

All patients = DF, DHF and OFIs (other febrile illness)

* Relative risk was not significant.

CHAPTER 6
DISCUSSION

Dengue hemorrhagic fever and dengue shock syndrome are the most severe form of virus infection to public health in many developing countries. They are remaining a major health problem in South East Asia, Central America and the Pacific region (3). The pathogenesis of the disease related to von Willebrand factor is not well understood.

This study have showed that von Willebrand factor antigen (VWF:Ag) and von Willebrand: Ristocetin cofactor activity (VWF:RcoF) in patients who developed shock (DSS) have significantly higher levels when compared with DF, DHF I, DHF II and OFIs patients. To our knowledge this is the first study to determine levels of VWF:Ag and VWF:RcoF in dengue illness and an association of increased VWF:Ag and VWF:RcoF levels with the severity of the patients with DHF. The finding of most interest is that the increased level of VWF:Ag and VWF:RcoF can be used as a predictive value in those patients with DHF who are about to develop a shock syndrome, especially on day of defervescence. According to these results, when the patients have VWF:Ag $\geq$ 210 %, they are at higher risk of contracting DSS than DF, DHF I, DHF II and OFIs patients (relative ratio = 15.6, 95% CI: 2.1-118.1, $p< 0.05$). And also the patients have VWF:RcoF $\geq$ 210 %, they are at higher risk of contracting DSS than DF, DHF I, DHF II and OFIs patients (relative risk = 12.6, 95% CI: 1.7-96.0, $p< 0.05$). Compared between DSS and DHF patients, the patients have VWF:Ag $\geq$ 210 % being at higher risk of contracting DSS (relative risk = 10.9, 95% CI: 1.5-81.7, $p < 0.05$). But the patients with VWF:RcoF $\geq$ 210 % having relative risk are not significant (RR = 7.0, 95% CI: 0.93-52.6, $p< 0.05$). These finding might be use as the prediction of DSS by von Willebrand factor antigen (VWF:Ag) and/or von Willebrand: Ristocetin cofactor activity (VWF:RcoF).

In DSS patients, the increase in VWF may be due to stress of the patients. The stress is due to the severity of disease itself and also stress to various procedures for diagnosis and management.

The multimer of von Willebrand factor in all dengue patients and OFIs do not show abnormal band on agar. This finding shows that the dengue patients have no the abnormal structure VWF multimer. There are no the conditions of acquired von Willebrand syndrome (AVWS) and thrombotic thrombocytopenic purpura (TTP) in dengue patients.

Rising Hct of ≥ 20 % is found in only 46.5 % of all DHF patients, whereas the percentage of rising Hct ≥ 20 % is found in 100 and 81.8 % of DSS and non-shock patients. Rising Hct of ≥ 20 % is not a sensitive criterion for DHF because Hct is not determined as frequent as needed in most cases and when there is rising Hct, increase rate of IV fluid or another effective colloidal solution may be used to lower the Hct level. In this study the patients with Hct ≥ 25 % are found in 80 % of DSS, and the relative risk of patients with Hct ≥ 25 % being at higher risk of contracting DSS is 7.7 (95% CI: 2.2-26.5, $p< 0.05$) and 11.5 (95% CI: 3.3-40.1, $p< 0.05$) of DHF patients and all patients.

Platelet count is inversely correlated with disease severe. About 82.5 % of all DHF have platelet count below 40,000 cells/µl, 24.2 % in DSS. The relative risk of patients with platelet count below 40,000 cells/µl being at higher risk of contracting DSS is 4.8 (95% CI: 1.1-21.3, $p< 0.05$) and 8.2 (95% CI: 1.9-36.7, $p< 0.05$) of DHF patients and all patients.

Prolonged APTT are found in 32.8 % of all DHF patients. The relative risk of patients with APTT ≥ 44 seconds being at higher risk of contracting DSS is 4.7 (95% CI: 1.3-16.8, $p< 0.05$) and 6.7 (95% CI: 1.9-24.1, $p< 0.05$) of DHF patients and all patients.

Prolonged PT are found in 24.6 % of all DHF patients. The relative risk of patients with PT ≥ 14 seconds being at higher risk of contracting DSS is 7.1 (95% CI: 2.1-24.7, $p< 0.05$) and 8.9 (95% CI: 2.5-31.5, $p< 0.05$) of DHF patients and all patients.

Prolonged TT are found in 28.7 % of all DHF patients. The relative risk of patients with TT ≥ 16 seconds being at higher risk of contracting DSS is 9.9

(95% CI: 2.3-42.8, $p< 0.05$) and 15.3 (95% CI: 5.3-44.4, $p< 0.05$) of DHF patients and all patients.

The prognosis of DHF depends on prevention or early treatment of shock. Once shock develops, the mortality may be as high as 44 % (85). The early recognition of the risk factors may be helpful in successful identification and management, analysis of the relative risk in this study may offer other clues useful in the detection of DSS.

In case of DSS the rising levels is higher than in DF and DHF grade I and II. If Hct is rising around 20%, they can compensate and have no vital signs or clinical evident of shock. The bone marrow suppression appears to result from the direct infection of hematopoietic progenitor cells (86) and bone marrow stromal cells by dengue virus (87) as well as the release of various hematodepressive cytokines during the dengue virus infection. Recently, it has been shown that cytokines, some of which could suppress hematopoiesis, were released into the circulation during the early acute febrile phase of dengue infection. These cytokines included tumor necrotic factor (TNF-α), interleukins (IL-2, IL-6, IL-8) and interferons (IFN-α, IFN-γ). As thrombocytopenia is found in DF and is a constant finding in DHF/DSS (2, 39). Our study was also observed the thrombocytopenia in all groups of dengue patients in either early or acute stage. The depression in the bone marrow observed in DHF in the acute stage may account for thrombocytopenia (88). In addition, dengue virus infection of megakaryocytes could also lead to an increase in the destruction of these cells (89) and some of which may result from the direct effect of dengue virus on endothelial cells. Funahara et al., have demonstrated an interaction between platelet and endothelial cells infected with dengue virus *in vitro* and suggested that some injury of vascular endothelial cells caused by dengue virus may give the blood circulating cells in the vessel to interact with collagen in the subendothelial layer and lead to the promotion of platelet aggregation and lysis of platelets resulting in thrombocytopenia (90). Therefore, both increase of platelet consumption and decrease of platelet production may cause thrombocytopenia during dengue virus infection.

CHAPTER 7
CONCLUSION

The purpose of this study was to assess the von Willebrand factor (VWF) in patients with dengue virus infection. The assessment of VWF in 21 dengue fever (DF), 30 dengue hemorrhagic fever (DHF) grade I, 33 DHF grade II, 10 dengue shock syndrome (DSS) and 7 other febrile illness (OFIs) was conducted. The results showed that the DHF patients who developed shock (DSS) had significantly higher levels of either von Willebrand factor antigen (VWF:Ag) or ristocetin cofactor activity (VWF:RcoF), functional test of von Willebrand factor when compared with the other group of patients ($p< 0.05$). Additionally, the multimer of von Willebrand factor did not show the abnormal band, unusually large VWF (ULVWF) multimers or the loss of high molecular weight (HMW) VWF mutimer on SDS-AgaroseGel Electrophoresis (SDS-AGE). This finding showed that the dengue patients have no the abnormal structure VWF multimer. There were no the conditions of acquired von Willebrand syndrome (AVWS) and thrombotic thrombocytopenic purpura (TTP) in dengue patients. The assessments of the association between laboratory results and the severity of the dengue disease found that the level of VWF:Ag $\geq$ 210 % being at higher risk of contracting DSS was 10.1 (95% CI: 5.3-44.4, $p< 0.05$) and 10.9 (95% CI: 1.5-81.7, $p< 0.05$) of DHF patients and all patients. In addition, VWF:RcoF $\geq$ 210 % being at higher risk of contracting DSS was 12.6 (95% CI: 1.7-96.0, $p< 0.05$) of all patients, but of DHF patients was not significant higher risk of contracting DSS (relative risk = 7.0, 95% CI: 0.93-52.6, $p< 0.05$).

For hematological outcomes, level of rising Hct $\geq$ 25 % and level of platelet count $\leq$ 40,000 cells/µl were higher risk of contracting DSS. In addition to the prolonged value of coagulation screening test, APTT $\geq$ 44, PT $\geq$ 14 and TT $\geq$ 16 seconds were higher risk of contracting DSS also.

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